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PRINCIPAL INVESTIGATOR: David M. Yurek, Ph.D.

CONTRACTING ORGANIZATION: University of Kentucky
Research Foundation
Lexington, KY 40506-0057

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David M. Yurek, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Kentucky
Research Foundation
Lexington, KY 40506-0057

E-Mail: dyure00@uky.edu

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13. ABSTRACT (Maximum 200 Words)

The etiology of Parkinson's disease is not known and may be related to several factors which include inheritable mutations (genetic), exposure to environmental toxins, and/or traumatic head injury. Our current research examines age-related changes in neurotrophic factor expression in Brown Norway/(Fischer 344 F1 hybrid ($F344BNF_1$)rats, and we have preliminary evidence that the young and aged nigrostriatal system responds differently to neurotoxic insult or mechanical injury, *i.e.*, young rats show a tendency to increase neurotrophic factor expression while aged rats do not. This is an important finding in the sense that the success of new therapies utilizing embryonic neurons or stem cells may be dependent on how well the implanted cells interact with the host neurotrophic environment. The studies proposed in this research project will further characterize the temporal expression of neurotrophic markers before and after neurotoxic insult or mechanical injury to the nigrostriatal system in young, middle-age, and old $F344BNF_1$ rats. The second part of this project will demonstrate that age differences in compensatory neurotrophic mechanisms that occur in the nigrostriatal system have a direct impact on the success of embryonic neurons implanted into the injured or denervated striatum.

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Introduction

The main hypothesis to be tested is whether or not molecular markers for neurotrophic factors and their receptors show a greater compensatory response to neurotoxic insult or injury in young brain than in older brain in the nigrostriatal system. Our most recent data shows during normal aging, there is an increased expression of factors that may be neurotrophic for dopamine neurons; however, only in young animals receiving unilateral lesions do we observe an increased expression of neurotrophic activity on the lesion side relative to the intact side of the brain. This is important from the standpoint that strategies which employ living cells to restore or release factors beneficial to injured brain regions may also require supplemental neurotrophic support for implanted cells, particularly if trophic mechanisms are diminished in the aging brain. We will test this hypothesis by implanting fetal dopaminergic grafts into the brains at various times relative to the lesion or injury, and then assess the integrity of the grafts. Based upon our preliminary studies, we hypothesize that grafts placed into the brains of young rats will show better graft survival and function than grafts placed into older brain. And lastly, we hypothesize that graft survival and function in aged rats with nigrostriatal injuries can be improved with supplemental treatments of neurotrophic factors.

Body

Over the three years of this project we have nearly completed a comprehensive study where we measured protein activity for two neurotrophic factors, BDNF and GDNF, following a neurotoxin-induced lesion of the aging nigrostriatal pathway. Initial studies were reported in Appendix 2. Table 1 (below) summarizes the data that has been completed to date. In the striatum of young animals, both BDNF and GDNF are up-regulated during the first 2 post-lesion weeks with BDNF remaining up-regulated for at least another two weeks. In old rats we do not observe a compensatory up-regulation of either of these neurotrophic factors at any time we looked during a 12 week post-lesion period; at the 2nd post-lesion week we even see a significant decline in striatal BDNF protein levels; this is consistent with a recent study by Collier *et al.* (Appendix 4) in which we report a significant decline of BDNF protein levels in aging MPTP-treated monkeys. We also see an immediate up-regulation of BDNF and GDNF in the ventral midbrain following the lesion and again BDNF protein levels remain elevated for at least 4 weeks; thus BDNF and FGF-2 protein (see above) show protracted elevations in the

Table 1

Relative changes of BDNF or GDNF at several post-lesion time points

(Young Rats, 4-5 months old)	3 Days	2 Weeks	4 Weeks	12 Weeks	16 Weeks
BDNF					
Lesioned striatum	n.s.	↑↑	↑↑	n.s.	n.s.
Lesioned midbrain	↑↑	↑↑	↑↑	n.s.	n.s.
GDNF					
Lesioned striatum	n.s.	↑↑	n.s.	n.s.	n.s.
Lesioned midbrain	↑	n.s.	n.s.	↓↓	↓
(Old Rats, 30 months old)					
BDNF					
Lesioned striatum	n.d.	↓↓	n.s.	n.s.	n.d.
Lesioned midbrain	n.d.	↑↑	n.s.	n.s.	n.d.
GDNF					
Lesioned striatum	n.d.	n.s.	n.s.	n.s.	n.d.
Lesioned midbrain	n.d.	n.s.	n.s.	n.s.	n.d.

↑↑ or ↓↓, significant difference vs. intact side; ↑ or ↓, approaching significance ($p \leq 0.07$); n.s. = not significant vs. intact side; n.d. = not determined

lesion ventral midbrain. We also observed a significant age-related reduction of GDNF protein values in both the intact and lesioned striata and ventral midbrain regions (see Appendix 2).

Studies in the last year of this project focused on the changes in activity of a third neurotrophic factor, fibroblast growth factor-2 (FGF-2 or bFGF). Most of the experiments were carried out in Brown Norway/Fischer 344 hybrid rats ($F344BNF_1$). The results from this study showed that unlike GDNF or BDNF, FGF-2 protein levels in the striatum and substantia nigra increased with age (Figures 1 & 2). We also measured dopamine levels in the striatum to determine the severity of the lesion. All the animals shown in figures 1 and 2 had >90% depletion of dopamine on the lesioned side. As you can see in figure 1, there was no significant difference in FGF-2 levels between the lesioned and intact striatum for any age group. On the other hand, FGF-2 protein levels increased with age regardless if the measures were taken from the lesioned or intact striatum. A similar finding was observed in the ventral midbrain (Figure 2); no significant difference in FGF-2 protein values was detected within each age group while FGF-2 protein values between each age group were statistically significant and increased with age.

Figure 1

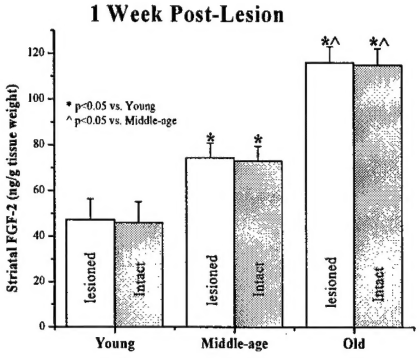
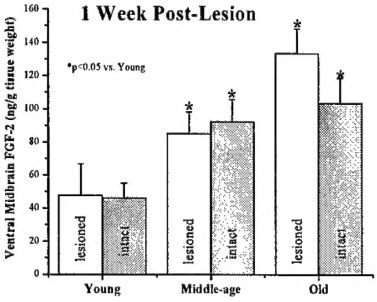


Figure 2



A curious finding occurred when we measured FGF-2 protein levels in the lesioned nigrostriatal pathway of young (4 month old) Sprague-Dawley rats. As already shown above, young (4 month old) $F344BNF_1$ rats did not show an increase expression of FGF-2 in the lesioned striatum or lesioned ventral midbrain at one week post-lesion. On the other hand, FGF-2 protein in lesioned ventral midbrain of Sprague-Dawley rats was nearly double the amount measured in the intact ventral midbrain (Figure 3). This is consistent with a previous report by Chadi *et al.*[11] in which a sustained increase in FGF-2 mRNA expression was observed in the lesioned substantia nigra of Sprague-Dawley rats 2 weeks after the lesion. On the other hand, we did not observe a significant change in FGF-2 protein in the denervated striatum at 1 or 2 weeks post lesion. This is no surprise given Chadi *et al.* (1994) also reported FGF-2 mRNA in the denervated striatum

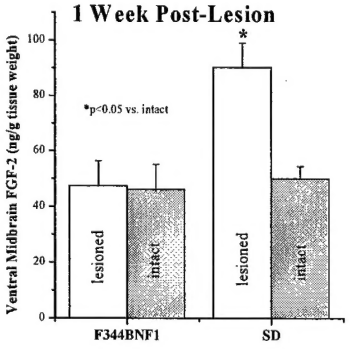


Figure 3

remained elevated for only 24 hours after the lesion. Moreover, FGF-2 protein remained up-regulated in the lesioned ventral midbrain 3 weeks post-lesion; at three weeks post-lesion, FGF-2 protein levels in the lesioned and intact ventral midbrain of young F344BNF$_1$ rats were statistically similar. This suggests that genetic differences within a species may play an important role for the regulation of neurotrophic factor activity, particularly in the neurodegenerative state.

In years 2-3 of this project we also performed a collaborative study with Dr. Timothy Collier in which we looked at changes in BDNF and GDNF in aging monkeys rendered hemiparkinsonian using the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). In this study we observed an age-related increase in neurotrophic activity; this was determined by taking soluble extracts from the brain tissue of aging monkeys and adding them to cultures of dopaminergic neurons (see Appendix 4 for details). We also determined that the increase of neurotrophic activity was not attributable to changes in GDNF or BDNF because these two factors showed either no change or decreases with age in monkeys. It is important to point out that in this monkey study the earliest time point neurotrophic factor activity was assessed in brain tissue was 12 weeks after the MPTP lesion. If you look at table 1, even in the rodent study we observed that the lesion-induced increases in BDNF or GDNF protein expression were only short-term events and that these two neurotrophic factors return to basal levels in the lesioned striatum or lesioned ventral midbrain by the 12th post-lesion week. Therefore, there is some consistency between the rodent and monkey studies; at least at the longer post-lesion time point. Given that our rodent study found an age-related increase in FGF-2 protein expression, it is unfortunate that we did not have these results prior to the commencement of the monkey study because we then could have incorporated measures of FGF-2 protein into the monkey study. When we compare the results from the rodent and monkey studies, we can speculate FGF-2 may be a likely candidate for one of the factors that mediates the increased neurotrophic activity in aging monkeys.

In situ hybridization studies were performed in collaboration with Dr. Kim Seroogy (co-investigator) and yielded several interesting age-related findings for the expression of dopaminergic or neurotrophic factor markers. For instance, we observed a significant age-related decline in the expression of tyrosine hydroxylase (TH) mRNA in the ventral midbrain. We also observed that the expression of erbB4 receptor mRNA showed a similar age-related decline; erbB4 receptor binds neuregulin and the neuregulins have been shown to exert neurotrophic support for dopaminergic neurons. Whether or not the decline in TH or erbB4 mRNA expression is directly related to an age-related loss of dopaminergic neurons has yet to be determined because of the numerous conflicting reports of age-related changes in dopaminergic neuron survival and function during the normal aging process[1-7, 9]. On the other hand, there does not appear to be significant age-related changes in the expression of mRNAs for other neurotrophic factor markers including BDNF, NT-3, trkB, or trkC during the normal aging process of the nigrostriatal system.

Experiments in year 2 were a continuation of the previous studies that showed age-related changes in neurotrophic factor expression following a neurotoxic lesion of the nigrostriatal pathway. Studies in the second year of this project focused on resolving an earlier problem and criticism related to our inability to determine the extent of the nigrostriatal lesion at time points immediately after the 6-OHDA lesion, *i.e.*, the post-

lesion time period between 3 days and 2 weeks post-lesion. During this post-lesion time interval behavioral measures [rotational or spontaneous motor tests] are unreliable because the nigrostriatal pathway typical undergoes progressive degeneration. On the other hand, the loss of dopamine occurs fairly rapidly after exposure to 6-OHDA. Given that our experimental design did not allow us to verify lesions using standard histological techniques because all brain tissue was subjected to an enzyme linked immunosorbent assay (ELISA), we devised a method of splitting the tissue sample for the ELISA analysis so that a portion of the sample could be used to detect dopamine levels using HPLC with electrochemical detection. Using this method we were able to correlate dopamine and neurotrophic factor protein levels in the same tissue sample (see Appendix 1). In most cases we observed lesions that produced a $\geq$75% reduction of striatal dopamine generally tend to increase BDNF and GDNF protein levels in the ipsilateral striatum of young animals. Lesions producing <60% reduction of striatal dopamine general do not affect BDNF or GDNF protein levels in the ipsilateral striatum in young animals. Similar to our previous studies, we do not observe significant changes in striatal BDNF or GDNF protein levels in aged animals regardless of the changes in striatal dopamine levels.

During the first year we attempted to produce a traumatic lesion of the nigrostriatal pathway; this was proposed as an alternate method for lesioning the nigrostriatal pathway. Unfortunately, as we reported in the year 1 progress report, the technique of lesioning the medial forebrain bundle [which contains nigrostriatal fibers] using a Scouten knife did not yield good lesions. Our next set of experiments used the Scouten knife as a means to disrupt the dopaminergic terminal fields and observe the consequential changes in neurotrophic factor expression. This technique yield similar results to the medial forebrain bundle knife cut lesions: lesions were minimal. We have abandoned the Scouten knife cuts as a means to induce a traumatic lesion to the nigrostriatal pathway or to the dopaminergic terminal fields in the striatum. Thus, the studies designed to examine the effect of a traumatic lesion never yielded positive results in terms of changing neurotrophic factor activity and/or producing robust lesions within the nigrostriatal pathway.

Transplants of fetal dopaminergic neurons implanted into the denervated striatum at several different post-lesion time points show that after 1 week post-lesion or 4 weeks post-lesion, dopamine grafts exhibit the best survival and functional reinnervation than grafts implanted immediately following the lesion or when implantation is delayed until the 12th post-lesion week. Moreover, the survival and fiber outgrowth of transplanted fetal dopamine neurons correlated well with the concomitant changes in BDNF and GDNF protein expression within the denervated striatum of young adult rats[10]. More information for this study can be found in Appendix 3. Because of a shortage of aged F344BNF$_1$ hybrid rats at the NIA aging colony during year 3 of this project, we were unable to implant grafts into the aged brain at various post-lesion time points and then compare graft survival and fiber outgrowth between young and aged rats. We are hoping we can continue these studies beyond the end of this project period.

Key Research Accomplishments

- Expression of BDNF and GDNF protein in the denervated striatum has now been correlated to the changes in striatal dopamine levels; this is important because we can now state with some degree of confidence that we have successful lesions at the earlier post-lesion time points.

- The expression of FGF-2 in rat brain increases with age

- Striatal neurotrophic activity increases with age in monkeys; however, this increase in neurotrophic activity is not attributable to changes in GDNF or BDNF.

- Genetic differences within species may be an important factor for regulating the expression of neurotrophic factors because young Sprague-Dawley rats show a protracted up-regulation of FGF-2 in the lesioned substantia nigra following a degenerative lesion of the nigrostriatal pathway while young F344BNF$_1$ hybrid rats do not show an up-regulation of FGF-2.

- We have determined in young rats that the most optimal time and place to implant dopamine grafts would be 1-2 weeks post-lesion into the substantia nigra; this observation is based upon data showing and elevation of 3 neurotrophic factors (BDNF, GDNF, and FGF-2) within ventral midbrain during this period.

Reportable Outcomes/Bibliography

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Results from this study help to provide preliminary data for a funded NIH grant entitled "Stem Cell Adaptability in Parkinson's Disease" [NS 050311].

Conclusions

Results from years 1-3 support the hypothesis neurotrophic factors are transiently elevated in components of the basal ganglia following a neurotoxic lesion of the nigrostriatal pathway of young adult rats. We have determined that protein levels of three dopaminergic neurotrophic factors [BDNF, GDNF, FGF-2] elevate within the ventral midbrain of young rats during the 1-2 week period post-lesion. Although this site alone is not the most optimal site for transplanted dopamine neurons in terms of producing functional recovery in the rat model of Parkinson's disease, placing grafts into this site will help us determine to what extent the expression of host neurotrophic factors play in the overall survival and functional integration of grafted dopaminergic neurons. A combination of intrastriatal and intranigral grafts at this same time point may yield the best results in terms of restoring motor function in lesioned rats.

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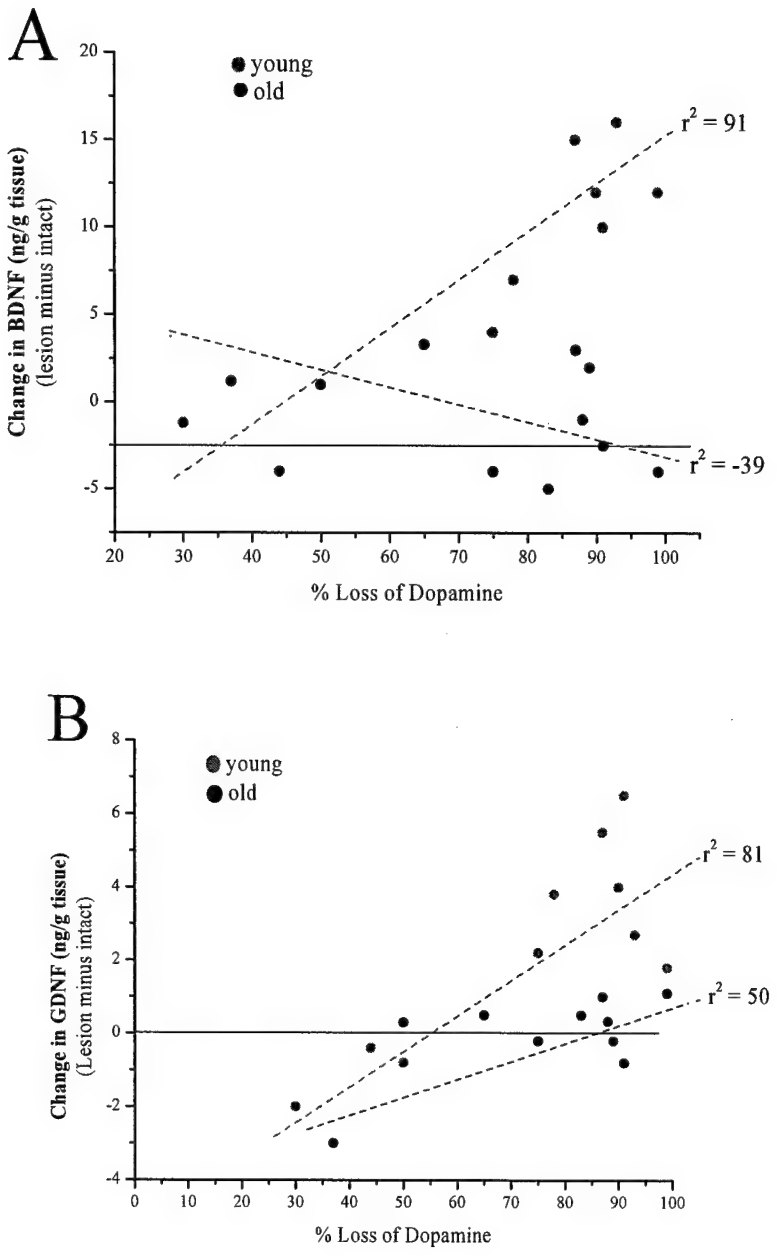
List of Personnel Employed by this Project:

Anita Fletcher-Turner
Charles Payne
Lee Dossett
Lixin Zhang
Stuart Lichtenberg
Jennifer Moore

Appendix 1



Correlation between the loss of striatal dopamine [extent of lesion] and the change in BDNF (A) or GDNF (B) protein levels in the denervated striatum at 2 weeks post-lesion. In (A) and (B) values above the solid black horizontal line represent "increases" in neurotrophic factor protein levels on the lesioned side relative to the intact side while values below the line represent "decreases" in neurotrophic factor protein levels. Dotted lines indicate the best linear fit for data in each age group [young = 4 month old, old = 30 month old].

Appendix 2

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Research report

Differential expression of GDNF, BDNF, and NT-3 in the aging nigrostriatal system following a neurotoxic lesion

David M. Yurek*, Anita Fletcher-Turner

Department of Surgery/Neurosurgery, University of Kentucky College of Medicine, Health Sciences Research Building, Lexington, Kentucky, KY 40536-0305, USA

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Abstract

Protein levels for brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and glial cell line-derived neurotrophic factor (GDNF) were measured in the striatum and ventral midbrain of young and aged Brown Norway/F344 F1 (F344BNF$_1$) hybrid rats following a unilateral 6-hydroxydopamine (6-OHDA) lesion of the nigrostriatal pathway. At 2 weeks post-lesion, protein levels of BDNF and GDNF were higher in the denervated striatum when compared to the intact striatum for young (4–5 months old) but not old (31–33 months old) rats. Interestingly, in old rats BDNF protein in the denervated striatum was significantly lower than that measured in the intact striatum. At the same time point BDNF protein levels in the ventral midbrain were higher on the lesioned versus intact side for both young and old rats while no significant side differences were detected for GDNF protein in the ventral midbrain of young or old rats. No significant differences in NT-3 protein levels were detected between the lesioned and intact sides for striatal or ventral midbrain regions in either young or old brain. While no significant age effects were detected for BDNF or NT-3 protein, young rats showed higher GDNF protein levels in both the striatum (lesioned or intact) and ventral midbrain (lesioned or intact) than old rats. These data show that two endogenous neurotrophic factors, BDNF and GDNF, are differentially affected by a 6-OHDA lesion in the aging nigrostriatal system with young brain showing a significant compensatory increase of these two factors in the denervated striatum while no compensatory increase is observed in aged brain. © 2001 Elsevier Science B.V. All rights reserved.

Theme: Development and regeneration

Topic: Neurotrophic factors: expression and regulation

Keywords: Neurotrophin-3; Brain-derived neurotrophic factor; Glial cell line-derived neurotrophic factor; Neurotrophic factor; Parkinson's disease; Brown/Norway F334 F$_1$ hybrid rats; 6-hydroxydopamine; Dopamine

1. Introduction

Animal models of Parkinson's disease are typically produced by lesioning the nigrostriatal pathway with various neurotoxins, e.g., 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) [18,37]. These lesions consequentially produce a hallmark symptom of Parkinson's disease, e.g., a loss of midbrain dopamine neurons. There is accumulating evidence that these lesions may also induced a compensatory cascade of neurotrophic activity within the nigrostriatal system as a physiologic response to the loss of dopamine neurons in young adult animals. This effect can be discerned from the results of the following studies. First, while extracts taken from the normal striatum enhance the survival and growth of cultured dopamine neurons [7,34], extracts taken from the lesioned striatum appear to provide more potent neurotrophic support. For example, striatal extracts taken from the lesioned striatum of young adult rats improve the survival of cultured dopamine neurons better than extracts taken from the normal striatum [3,21]. This effect has been extended to human dopamine neurons: cultures incubated with extracts from the caudate/putamen of patients with Parkinson's disease contained more tyrosine hydroxylase immunoreactive neurons than extracts obtained from aged controls [4]. Hida et al. demonstrated that striatal extracts taken from the lesioned striatum have stronger effects to hasten the differentiation of PC12D cells, promote neurite

*Corresponding author. Tel.: +1-859-257-8219; fax: +1-859-323-6343.

E-mail address: dyure00@pop.uky.edu (D.M. Yurek).

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outgrowth, cell enlargement, and expression of voltage-dependent cation channels when compared to the effects of extracts taken from the normal striatum [13]. More recently, specific neurotrophic factors native to the striatum have been shown to increase following a neurotoxic lesion of the nigrostriatal pathway. In young adult rats with unilateral 6-OHDA lesions, brain-derived neurotrophic factor (BDNF) protein levels are significantly elevated in the lesioned striatum and lesioned ventral midbrain when compared to BDNF protein levels in the same brain regions on the intact side [41,43].

Recent studies have provided evidence that the increase in neurotrophic activity in the denervated striatum is not consistent across the age of the lesioned animals. Ling et al. recently reported that the trophic activity of tissue extracts taken from the lesioned striatum of rats is inversely correlated to the age of the rat [20]. Similarly, Kaseloo et al. reported that striatal extracts taken from the injured striatum of aged rats possessed a diminished capacity for inducing neurite outgrowth in cultures containing a dopamine-producing neuroblastoma cell line [16]. Our recent study showed a compensatory increase of BDNF in the lesioned striatum 4 weeks after the lesion in young but not old rats [41]. The results of these studies suggest young and old brain may respond differently to neurodegenerative events: old brain shows a diminished capacity to elicit compensatory neurotrophic mechanisms. This area of research has been relatively overlooked in animal models of Parkinson's disease.

The purpose of this study was to further characterize how protein levels for three different neurotrophic factors [BDNF, neurotrophin-3 (NT-3), and glial cell line-derived neurotrophic factor (GDNF)] are affected by a neurotoxic lesion of the nigrostriatal pathway in both young and aged rats.

2. Material and methods

2.1. Animals

Young (4–5-month-old, $n=21$) and old (31–33-month-old, $n=14$) male Brown Norway/F344 F1 hybrid rats ($F344BNF_1$) rats were obtained from the NIA Aging Colony. Animals were housed in environmentally regulated rooms and had free access to food and water for the duration of the study. All animal procedures were conducted in strict compliance with approved institutional protocols, and in accordance with the provisions for animal care and use described in the 'Guide for the Care and Use of Laboratory Animals' (NIH publication No. 86-23, NIH, 1985).

2.2. 6-Hydroxydopamine lesions

Male $F344BNF_1$ rats in each age group were given unilateral 6-hydroxydopamine (6-OHDA) lesions of the nigrostriatal pathway; 6-OHDA (Sigma) was dissolved in 0.9% saline (containing 0.2% ascorbic acid) at a concentration of 2.0 μg/μl and stereotactically injected into the nigrostriatal pathway of anesthetized rats at a rate of 1.0 μl/min for 3 min. Each rat received two injections of 6-OHDA: one in the vicinity of the medial forebrain bundle (AP −4.3, ML 1.2, DV −7.5) and the other in the rostral pars compacta of the substantia nigra (AP −4.8, ML 1.5, DV −7.5); all coordinates reported in this study represent millimeter adjustments from bregma (AP, ML) and below the dural surface (DV) with the top of the skull in a flat position. This technique routinely produces complete lesions of A9 and A10 midbrain regions, and near-complete denervation of dopaminergic fibers innervating the ipsilateral striatum [33].

2.3. Quantification of neurotrophic factors by an enzyme-linked immunosorbent assay (ELISA)

Animals were euthanatized 2 weeks after the 6-OHDA lesion. Brains were removed, the striatal and substantia nigra/ventral tegmental area (SN/VTA) brain regions were dissected on ice, and the samples were then stored at −80°C. Subsequently, each tissue sample was homogenized in 400-μl volumes of homogenate buffer [400 mM NaCl, 0.1% Triton-X, 2.0 mM EDTA, 0.1 mM benzethonium chloride, 2.0 mM benzamidine, 0.1 mM PMSF, Aprotinin (9.7 TIU/ml), 0.5% BSA, 0.1 M phosphate buffer, pH=7.4]. The homogenate was centrifuged for 10 min at 10 000×g at 4°C. The homogenate was divided into 100-μl duplicate samples and neurotrophic factor content was determined using an antibody sandwich format: extracted neurotrophic factors from each sample were captured with a monoclonal antibody against BDNF, GDNF, or NT-3; the captured BDNF was then bound to a second, specific, polyclonal antibody (pAb) against BDNF, GDNF, or NT-3. After washing, the amount of specifically bound pAb was detected using a species-specific anti-IgY antibody conjugated to horseradish peroxidase (HRP) as a tertiary reactant. Unbound conjugate was removed by washing and, following an incubation period with a chromogenic substrate, the color change was measured in a microplate reader (450 nm). The amount of neurotrophic factor was proportional to the color change generated in an oxidation–reduction reaction.; the Promega E_{max}™ ImmunoAssay System was used for the detection of all three neurotrophic factors. The reliability of the neurotrophic factor measures ranged from 97 to 99% based upon regression analysis.

2.4. Statistical analysis

Comparison of side differences (lesion vs. intact) for neurotrophic factor protein levels were made using a paired t-test for each age group. Analysis of variance

(ANOVA) was used to analyze age differences in the data. The alpha level was set to 0.05.

3. Results

3.1. NT-3

There was no significant effect of age on NT-3 protein levels in the intact or lesioned striatum [$F(1,16)=0.27$, $P>0.05$], or in the lesioned or intact ventral midbrain [$F(1,16)=0.89$, $P>0.05$]. Neurotrophin-3 levels in the lesioned striatum were not significantly different from the intact striatum for either young ($P=0.70$) or old rats ($P=0.63$). Similarly, no significant differences in NT-3 were detected between the lesioned and intact ventral midbrain for young ($P=0.51$) or old rats ($P=0.14$). These data are summarized in Fig. 1.

3.2. BDNF

Fig. 2 shows a comparison of mean BDNF values in the lesioned or intact striatum of young or old rats. Two weeks after the lesion young rats show a higher level of BDNF protein in the lesioned striatum when compared to the intact striatum ($P=0.01$). On the other hand, in old rats BDNF protein levels are significantly lower in the lesioned striatum than in the intact striatum ($P<0.001$). A significant effect of age on striatal BDNF levels [$F(1,47)=6.32$, $P=0.016$] was detected between the lesioned striatum of young and old rats ($P<0.05$) while the effect of age was not significant for the intact striatum ($P>0.05$).

Comparison of BDNF protein levels in the ventral midbrain of young and old rats show significantly higher levels of BDNF protein in the lesioned vs. the intact side for both young ($P=0.01$) and old ($P=0.038$) rats. There

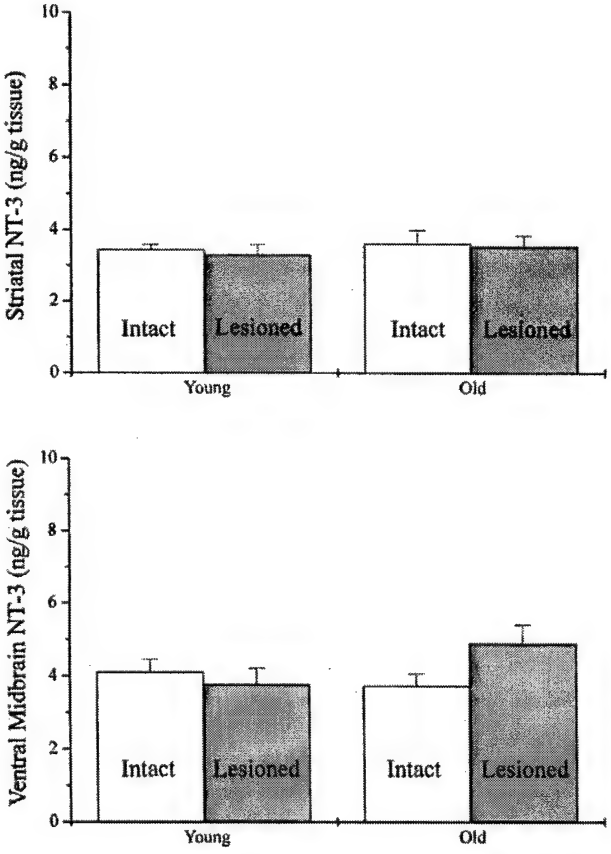

Fig. 1. NT-3 protein levels (ng/g tissue) in the striatum (top) or ventral midbrain (bottom) of young ($n=5$, 4–5-month-old) or old ($n=5$, 31–33-month-old) F344BNF$_1$ rats. Animals were given a unilateral 6-OHDA lesion and sacrificed 2 weeks later. Tissue was dissected from the striatum and ventral midbrain from both the lesioned and intact hemispheres and subjected to ELISA analysis.

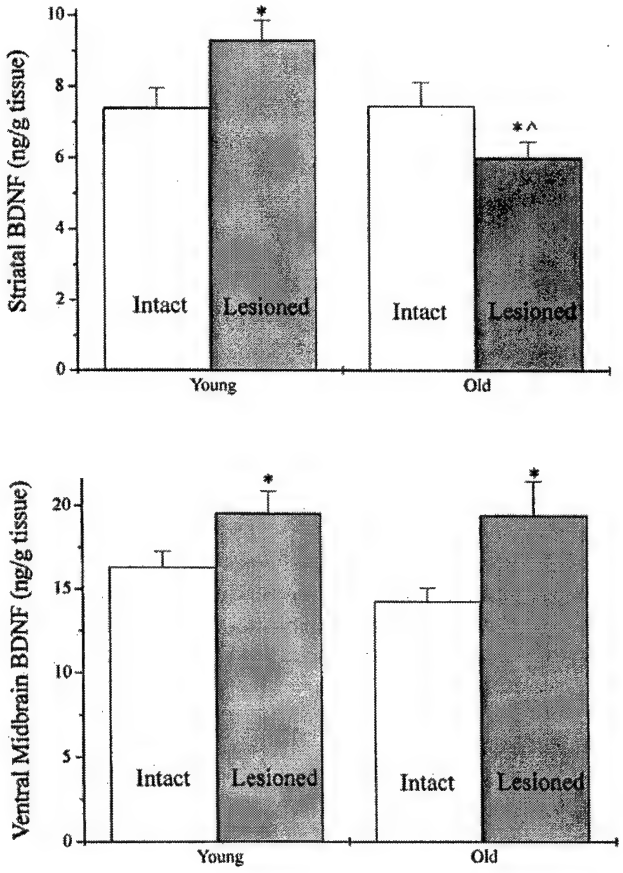

Fig. 2. BDNF protein levels (ng/g tissue) in the striatum (top) or ventral midbrain (bottom) of young ($n=16$, 4–5-month-old) or old ($n=9$, 31–33-month-old) F344BNF$_1$ rats. Animals were given a unilateral 6-OHDA lesion and sacrificed 2 weeks later. Tissue was dissected from the striatum and ventral midbrain from both the lesioned and intact hemispheres and subjected to ELISA analysis. *$P<0.05$, intact vs. lesioned; ^$P<0.05$, young vs. old.

was no significant effect of age on BDNF protein levels in ventral midbrain on either side [$F(1,47)=0.57$, $P=0.452$].

3.3. GDNF

The results for GDNF analysis are shown in Fig. 3. Two weeks after the lesion young rats showed higher GDNF protein levels in the lesioned striatum than in the intact striatum ($P<0.001$). In old rats GDNF protein levels in the lesioned and intact striatum were not statistically different from one another ($P=0.98$). Analysis of variance revealed a significant effect of age on GDNF protein levels in the striatum [$F(1,49)=28.14$, $P<0.001$]. The lesioned striatum of young rats contained higher levels of GDNF protein than the lesioned striatum of old rats ($P<0.05$), and the intact striatum of young rats contained higher levels of GDNF protein than the intact striatum of old rats ($P<0.05$).

We observed only a slight but non-significant increase of GDNF protein on the lesioned side in the ventral midbrain of young rats ($P=0.41$). Similarly, midbrain levels of GDNF in the lesioned and intact sides were not significantly different from one another ($P=0.80$). The effect of age on ventral midbrain GDNF levels was significant [$F(1,49)=21.45$, $P<0.001$]. The lesioned ventral midbrain of young rats contained higher levels of GDNF protein than the lesioned ventral midbrain of old rats ($P<0.05$), and the intact ventral midbrain of young rats contained higher levels of GDNF protein than the intact ventral midbrain of old rats ($P<0.05$).

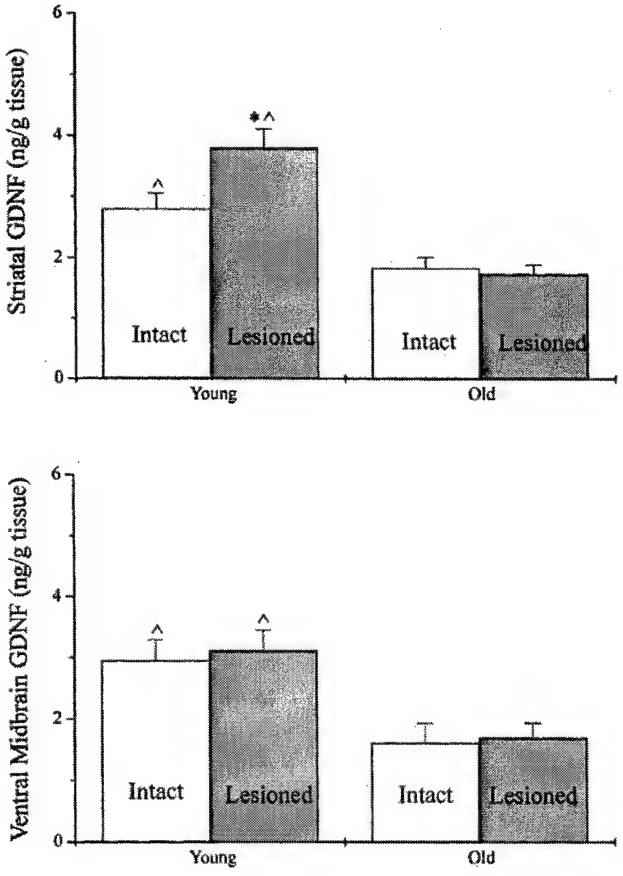

Fig. 3. GDNF protein levels (ng/g tissue) in the striatum (top) or ventral midbrain (bottom) of young ($n=16$, 4–5-month-old) or old ($n=9$, 31–33-month-old) F344BNF$_1$ rats. Animals were given a unilateral 6-OHDA lesion and sacrificed 2 weeks later. Tissue was dissected from the striatum and ventral midbrain from both the lesioned and intact hemispheres and subjected to ELISA analysis. *$P<0.05$, intact vs. lesioned; ^$P<0.05$, young vs. old.

4. Discussion

The results of this study provide evidence that the expression of three different neurotrophic factors within the mesostriatal system are differentially affected by a neurotoxic lesion of the nigrostriatal pathway during aging. In young rats the expression of two neurotrophic factors, BDNF and GDNF, increase within the denervated 2 weeks following a nigrostriatal lesion while NT-3 protein levels in the denervated striatum did not change significantly. In aged rats protein expression of BDNF was significantly reduced in the denervated striatum while GDNF and NT-3 did not change significantly. Protein levels of BDNF in the lesioned ventral midbrain were significantly higher than those observed in the intact ventral midbrain in both young and aged rats. Glial cell line-derived neurotrophic factor was the only one of the three proteins studied to show an age-related reduction in both the lesioned and intact mesostriatal system of F344BNF$_1$ rats.

Glial cell line-derived neurotrophic factor is a distant member of the TGF-β family of neurotrophic factors and is expressed in the substantia nigra and striatum, as well as other brain regions, in both the developing and adult brain of rats and humans [25,26,31]. The functional receptor for GDNF is a two-component receptor complex that consists of a ligand binding GDNF family receptor, GDNFR-α1 or GDNFR-α2, and the receptor protein kinase ret [10,15,35,36]. In rats, dopamine neurons express both GDNFR-α mRNA and ret mRNA during development and throughout adulthood while only GDNFR-α mRNA is expressed in the ventral striatum during development [22]. The ret protein has been identified immunohistochemically to be on dopamine neurons in adult rat brain [22]. Thus the functional receptor of GDNF appears to be present in dopamine neurons throughout the lifetime of rats. Injury to dopamine neurons or the striatum can elicit changes in the expression of GDNF or its receptor. For instance, while GDNF mRNA expression is not observed in the striatum of normal adult rats [32], its expression in the striatum can

be induced by status epilepticus in motor and limbic brain regions [27]. In mice, MPTP treatment does not change the expression of GDNF mRNA in the denervated striatum [14] while mechanical injury to the striatum elicits an increased expression of GDNF mRNA [19]. Ischemic brain injury via occlusion of the middle cerebral artery can induce GDNFR-α1 and ret in the striatum [17]. In the present study we observe that GDNF protein is increased in the denervated striatum of young rats. This increased expression of GDNF protein, GDNF mRNA, and GDNFR mRNA in the denervated striatum may be a compensatory neurotrophic response to the loss of striatal afferents and/or direct injury to the striatum.

Previous studies have established that the neurotrophins BDNF and NT-3, along with their receptors [trkB and trkC], are expressed within the mesostriatal system during development and throughout adulthood [11,24,29,30]. The profuse expression of these neurotrophins and their receptors in the ventral midbrain during development suggests that these two neurotrophins may play an important role for the differentiation, maturation, and target innervation of dopamine neurons. The sustained expression of these neurotrophins and their receptors in adult brain suggests a role for the maintenance and repair of the mesostriatal system throughout the lifetime of the organism. Injury to the mesostriatal system alters the expression of neurotrophins and neurotrophin receptors in young adult rodents. For instance, transection of the medial forebrain bundle induces an up-regulation of trkB protein in the ipsilateral striatum [8]. The expression of the full-length form of trkB mRNA in the denervated striatum is up-regulated at 2 weeks [23] and 8 weeks [42] after the nigrostriatal pathway is neurotoxically lesioned with 6-OHDA. Following a mechanical injury to mouse striatum, the expression of BDNF mRNA and the truncated, but not full-length, form trkB mRNA are increased in the injured striatum [39]. The results of the present study along with those reported by Zhou et al. [43] show an increase of BDNF in the denervated striatum in young adult rats. Taken together, the results of the aforementioned studies provide convincing evidence that the expression of BDNF and trkB receptor increase as a consequence of striatal injury or a neurotoxic lesion of the nigrostriatal pathway of young adult rodents. On the other hand, we did not observe a change in striatal NT-3 protein levels 2 weeks following a 6-OHDA lesion in either young or old rats, nor does the expression of trkC mRNA change significantly in the denervated striatum of young adult rats 2 weeks after a 6-OHDA lesion [23]; it is noteworthy that unlike the increase of trkB mRNA expression 8 weeks after a 6-OHDA lesion, the expression of trkC mRNA is actually decreased in the denervated striatum of young rats [42]. In this study we observe the two neurotrophins, BDNF and NT-3, are differentially expressed in the denervated striatum of young adult rats in response to a lesion of the nigrostriatal pathway. In aged rats, however, we provide evidence that at least three neurotrophic factors [BDNF, NT-3, or GDNF] do not show a compensatory increase following a 6-OHDA lesion of the nigrostriatal pathway.

The lack of a compensatory increase in BDNF or GDNF within the lesioned striatum of aged rats is consistent with other neurotrophic factors in other denervated brain regions. For example, following a medial septal lesion only young rats demonstrated significant increases in sympathetic sprouting and NGF-like activity in the hippocampus [28]; this suggests that the age-related deficit in sympathetic sprouting may result from an attenuated neurotrophic response to hippocampal denervation, similar to what we observe in the denervated striatum of old rats. It still remains unclear why compensatory neurotrophic mechanisms may diminish with age.

Interestingly, we observed better survival, fiber outgrowth, and functional reinnervation for fetal ventral mesencephalic tissue transplants when the tissue is implanted 1 or 4 weeks after a 6-OHDA lesion rather than 1 week before the 6-OHDA lesion [40]. Not surprisingly, the post-lesion period when transplant development is robust also coincides with the post-lesion period when at least two neurotrophic, BDNF and GDNF, are increased in the denervated striatum. The expression of other neurotrophic factors, e.g., bFGF [5], are increased in the denervated striatum immediately following a nigrostriatal pathway lesion. A critical period for the survival of transplanted dopamine neurons occurs during the first 4 days immediately following implantation [9]. During this critical period, fetal neurons implanted into the intact striatum 1 week prior to a 6-OHDA lesion would not be exposed to the same enriched neurotrophic environment as those implanted after the lesion. The results of this study strongly suggest that the striatal environment of the intact striatum may not be as conducive to the survival, fiber outgrowth, and function of transplants as is the lesioned striatum. This is consistent with the results of the present study and with the results of previous studies that demonstrated prior injury to the striatum improves the survival of fetal dopamine implants [1,2]. Up-regulation of neurotrophic activity in the injured or denervated striatum of young animals may actually be beneficial to the survival and functional reinnervation of implanted donor cells. In old rats, however, we did not observe an increase of BDNF or GDNF protein levels in the denervated striatum. This may be a significant finding in terms of the success that fetal cell implants may have in aged brain. The recent study completed by Collier et al. [6] provides compelling evidence that transplants in the aged brain show a poorer survival rate and less functional compensation than transplants into young brain; therefore the age of the transplant recipient may be an important determinant for the survival and/or functional effects of fetal mesencephalic transplants. Furthermore, a recently completed clinical trial using dopamine neuron implants in Parkinson's patients concluded that patients under 60 years of age exhibited

statistically significant clinical benefits from transplants while patients older than 60 years of age did not [12]. The results of the present study provide initial evidence that the denervated striatum of young rats may become neurotrophically enriched following a degenerative lesion of the nigrostriatal pathway and thus provide a more nurturing environment for transplant development than in aged brain.

In the present study we observed significantly lower levels of BDNF in the lesioned striatum than in the intact striatum of old rats at the 2-week post-lesion time point. In a previous study we reported that BDNF protein levels in the lesioned and intact striatum of aged rats were not significantly different from one another at the four week post-lesion time point [41]. The results of the present study are not entirely inconsistent with our previous report, however. Previously we reported that at the four week post-lesion time point, the most severely lesioned old rats tended to show a greater reduction of BDNF in the denervated striatum than old rats with less severe lesions.

While previous studies have shown a reduction in BDNF mRNA labeling within the substantia nigra following a lesion of the dopamine cell bodies [29,30,38], we observe an increase of BDNF protein in the lesioned ventral midbrain of both young and old rats. Seroogy et al. [29,30] report approximately 20% of BDNF mRNA labeling in the ventral mesencephalon occurs in non-dopaminergic cell bodies, and Venero et al. [38] report a continued expression of BDNF mRNA labeling within the ventral tegmental area and pars lateralis of the substantia nigra following a 6-OHDA lesion. Taken together, these data provide evidence that BDNF mRNA is localized to dopaminergic and non-dopaminergic cell bodies within the ventral mesencephalon. The increase of BDNF protein within the lesioned ventral midbrain may result from a local compensatory reaction to the lesion by non-dopaminergic neurons and a concomitant accumulation of BDNF that might occur after dopamine neurons, which normally bind and take up BDNF, are lost as a result of the lesion. The increase in BDNF protein in the lesioned ventral midbrain of young animals are consistent with the increase of BDNF content in the lesioned substantia nigra observed 2 weeks [43] and 4 weeks [41] after the lesion.

Because we were unable to assess the degree of the 6-OHDA lesion prior to obtaining our samples, it is possible that our final analysis of the data included samples taken from animals with incomplete or poor lesions. The short interval between the time the animals were lesioned and the time the animals were sacrificed did not allow us to use conventional tests to accurately assess lesion severity, e.g., amphetamine- or apomorphine-induced rotational behavior. In addition, no tissue samples were available for the determination of dopamine content in either the substantia nigra or striatum because all samples were used for ELISA analysis. This may be one explanation why the difference in BDNF protein levels between the lesioned and intact striata at 2 weeks post-lesion was not as great as that observed 4 weeks post-lesion [41]; at 4 weeks post-lesion, animals with no evidence of a lesion were excluded from the study. Another explanation for this phenomenon is that BDNF protein levels in the denervated striatum increase progressively following a nigrostriatal pathway lesion. In order to determine whether the increase of lesion-induced neurotrophic activity is progressive, transient, or both, the time course of this phenomenon needs to be more fully characterized.

In conclusion, neurotoxic lesion of the nigrostriatal pathway affects the expression of several specific neurotrophic factors differentially, and the expression is also dependent upon the age of the animal. Both BDNF and GDNF protein levels in the lesioned striatum are increased 2 weeks following a 6-OHDA lesion whereas these same two neurotrophic factors do not show a compensatory increase in the lesioned striatum of old rats. The expression of GDNF shows an age-related decline in both the lesioned and intact striatum. The results of this study provide evidence that young animals show an enhanced neurotrophic response to a neurotoxic lesion that is not observed in older animals. The differential expression of these neurotrophic factors may have a direct effect on the success of therapies which use cellular implants to correct neurodegenerative disorders, particularly if the cellular implants are dependent upon neurotrophic factors for differentiation, survival, and the maintenance of function.

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Appendix 3

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Research report

Temporal changes in the neurotrophic environment of the denervated striatum as determined by the survival and outgrowth of grafted fetal dopamine neurons

David M. Yurek*, Anita Fletcher-Turner

Department of Surgery/Neurosurgery, University of Kentucky College of Medicine, Health Sciences Research Building, Lexington, KY 40536-0305, USA

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Abstract

There is growing evidence that the neurotrophic environment of the denervated striatum may change with time following a lesion of the nigrostriatal pathway in young adult rats. To test this hypothesis, we implanted fetal dopamine grafts into the striatum at several different time points relative to the nigrostriatal pathway lesion and allowed the grafts to integrate with the host for a period of 1 month; subsequently, we observed the function and morphology of the dopamine grafts. Fetal grafts were implanted at the following time points relative to the lesion: 1 week before (−1 Week), at the same time (Week 0), 1 week after (1 Week), 4 weeks after (4 Weeks), or 12 weeks after (12 Weeks). Amphetamine-induced rotational behavior was assessed 4 weeks after grafting for all groups. Rotational scores indicate that grafts for the 1 Week group showed the greatest reversal of amphetamine-induced rotational behavior that was also significantly greater than the scores for the −1 Week group. Morphological analysis revealed that grafts in the Week 0, 1 Week and 4 Weeks groups showed a significantly larger area of tyrosine hydroxylase-positive (TH+) fiber outgrowth than in the −1 Week group, while fiber outgrowth for the 12 Weeks group was significantly lower than for the 1 Week group. Cell count analysis for TH+ neurons within the graft indicate a significantly greater number of TH+ neurons in grafts for the 1 Week group than in grafts for the −1 Week. The results of this study suggest that neurotoxic lesions may induce a compensatory increase in neurotrophic activity within the denervated striatum of young rats that is conducive to the survival and outgrowth of fetal dopamine grafts. These data also correlate well with reports that the expression of several specific dopaminergic neurotrophic factors within the striatum increase following a neurotoxic lesion of the nigrostriatal pathway in young adult rats. © 2002 Elsevier Science B.V. All rights reserved.

Theme: Development and regeneration

Topic: Transplantation

Keywords: Dopamine; Neural transplantation; Parkinson's disease; Neurotrophic factor; Glial cell line-derived neurotrophic factor; Brain-derived neurotrophic factor; 6-Hydroxydopamine; Rodent; Striatum

1. Introduction

Several studies have provided evidence that neurotoxic and ablative lesions of the nigrostriatal pathway induce an increase in neurotrophic activity within the denervated striatum. Chadi et al. [7] demonstrated an immediate increase of basic fibroblast growth factor (bFGF) mRNA and immunoreactivity in the denervated striatum following a 6-hydroxydopamine (6-OHDA) lesion of the nigrostriatal pathway. Specific neurotrophic factors, e.g. brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), increase expression within the denervated striatum following a 6-OHDA lesion in young adult brain [32,33,39]. The increase in striatal neurotrophic activity following a nigrostriatal pathway lesion is further substantiated by evidence that striatal extracts taken from the denervated striatum enhance the survival of cultured dopamine neurons [5,25]. The specific neurotrophic factors that increase their expression in the denervated striatum, e.g. bFGF, BDNF, and GDNF, have

*Corresponding author. Tel.: +1-859-257-8219; fax: +1-859-323-6343.

E-mail address: dyure00@uky.edu (D.M. Yurek).

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been shown to provide potent neurotrophic support to dopamine neurons in vitro [12,14,17,18,20,22,24,38]. The increased expression of neurotrophic factors within the denervated striatum may be an underlying mechanism that supports differentiation, survival, and functional outgrowth of grafted embryonic neurons. Previous studies have shown that fetal dopamine grafts supplemented with neurotrophic factors can successfully improve the survival and function of the grafts [3]; in particular, treatment of fetal dopamine grafts with exogenous BDNF and GDNF before or after implantation of the grafts improves the function and survival [1,26–28,31,34,37].

The purpose of the present study was to compare the survival, fiber outgrowth, and function of fetal dopamine grafts when these grafts are implanted into the lesioned or intact striatum of young adult rats, and determine whether lesioned-induced neurotrophic activity may be beneficial to graft development and function.

2. Material and methods

2.1. Animals

A total of 54 young (4–5 months old) male Sprague–Dawley rats were obtained from Harlan Farms and used in this study. Animals were housed in environmentally regulated rooms and had free access to food and water for the duration of the study. All animal procedures were conducted in strict compliance with approved institutional protocols, and in accordance with the provisions for animal care and use described in the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, 1985).

2.2. Ventral mesencephalic tissue grafts

Recipient animals were anesthetized with halothane (1.0–1.5% mixture with air) and placed in a stereotaxic apparatus. At the same time, the ventral mesencephalon was dissected from E14 fetuses obtained from time-pregnant Sprague–Dawley rats (Harlan Farms) and stored individually in a cold, sterile, calcium-magnesium free buffer (CMF: 0.15 M NaCl, 8.0 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM $KHPO_4$, 26.0 mM $NaHCO_3$, 0.1% glucose, 100 mg/ml streptomycin, 2.5 mg/ml fungizone, pH 7.2). The ventral mesencephalon from a single fetus was drawn into the blunt end of a 22-gauge spinal needle and stereotaxically placed into the denervated striatum of the recipient animal at the following coordinates: AP +0.5, ML +2.5, DV −5.5. Animals received grafts according to the following schedule: for the −1 Week group (n=8), grafts were implanted into the intact striatum 1 week before the ipsilateral nigrostriatal pathway was lesioned; for the Week 0 group (n=6), each animal received a unilateral 6-OHDA lesion and immediately thereafter a graft was implanted into the ipsilateral striatum; for the 1 Week (n=9), 4 Weeks (n=8), and 12 Weeks (n=6) groups, grafts were placed into the lesioned striatum 1, 4, or 12 weeks after the 6-OHDA lesion, respectively.

2.3. 6-Hydroxydopamine lesions

All rats were given unilateral 6-hydroxydopamine (6-OHDA) lesions of the nigrostriatal pathway; 6-OHDA (Sigma) was dissolved in 0.9% saline (containing 0.2% ascorbic acid) at a concentration of 3.0 μg/μl and stereotaxically injected into the nigrostriatal pathway of anesthetized rats at a rate of 1.0 μl/min for 2 min. Each rat received two injections of 6-OHDA: one in the vicinity of the medial forebrain bundle (AP −4.4, ML 1.2, DV −7.5) and the other in the rostral pars compacta of the substantia nigra (AP −5.3, ML 2.0, DV −7.5); all coordinates reported in this study represent millimeter adjustments from bregma (AP, ML) and below the dural surface (DV) with the top of the skull in a flat position. This technique routinely produces complete lesions of dopamine neurons in the A9 and A10 midbrain regions, and near complete denervation of dopaminergic fibers innervating the ipsilateral striatum.

2.4. Quantification of neurotrophic factors by an enzyme-linked immunosorbent assay (ELISA)

A total of 17 rats were euthanatized either 3 days (n=10) or 12 weeks (n=7) after receiving a unilateral 6-OHDA lesion. Brains were removed, the striatal and ventral midbrain brain regions of both hemispheres were dissected on ice and the samples were then stored at −80 °C. Subsequently, each tissue sample was homogenized in 300-μl volumes of homogenate buffer (400 mM NaCl, 0.1% Triton-X, 2.0 mM EDTA, 0.1 mM benzethonium chloride, 2.0 mM benzamidine, 0.1 mM PMSF, Aprotinin (9.7 TIU/ml), 0.5% BSA, 0.1 M phosphate buffer, pH 7.4). The homogenate was centrifuged for 10 min at 10,000×g at 4 °C. The homogenate was divided into 100-μl duplicate samples and neurotrophic factor content was determined using an antibody sandwich format: extracted neurotrophic factors from each sample were captured with a monoclonal antibody against BDNF or GDNF and the captured neurotrophic factor was then bound to a second, specific, polyclonal antibody (pAb) against BDNF or GDNF. After washing, the amount of specifically bound pAb was detected using a species-specific anti-IgY antibody conjugated to horseradish peroxidase (HRP) as a tertiary reactant. Unbound conjugate was removed by washing and, following an incubation period with a chromogenic substrate, the color change was measured in a microplate reader (450 nm). The amount of neurotrophic factor was proportional to the color change generated in an oxidation–reduction reaction; the Promega E_{max}™ ImmunoAssay System was used for the detection of both neurotrophic factors. The reliability of the neuro-

trophic factor measures ranged from 97 to 99% based upon regression analysis. We chose not to examine the expression of BDNF or GDNF at time points between 3 days and 12 weeks post-lesion because these studies were performed earlier [32,33].

2.5. Rotational behavior

Amphetamine-induced rotational behavior was tested in all treatment groups 4 weeks after grafting. Rotational behavior was induced by a systemic injection of amphetamine (5.0 mg/kg, i.p.). Rats were placed inside opaque 16-inch diameter cylindrical chambers which were positioned directly beneath a video camera. The video camera was connected to a Videomex V image motion computer system (Columbus Instruments, Columbus, OH). The total number of 360° clockwise or counterclockwise rotations was measured during each 90-min test session. No post-lesion, pre-graft rotational scores are reported because three of the five treatment groups received fetal grafts at time points before the 6-OHDA lesions were fully developed.

2.6. Immunohistochemical technique

Rats were sacrificed at the end of the 6th postgraft week for all treatment groups. All rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with ice-cold saline followed by 4% paraformaldehyde. The brains were post-fixed overnight in 4% paraformaldehyde and placed in 30% sucrose. Brain sections (40 μm) were cut on a sliding microtome and stored in cryoprotectant at −20 °C [30]. For immunohistochemical detection of tyrosine hydroxylase (TH) free-floating sections were incubated overnight in mouse antisera containing a monoclonal antibody against TH (1:8000; Chemicon). The sections were then incubated in an affinity-purified biotinylated goat anti-mouse IgG secondary antibody (1:800, Chemicon, Temecula, CA) and then incubated in an avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA). Staining was completed by placing the sections in 0.003% H_2O_2 that contained diaminobenzidine chromogen to visualize the peroxidase-catalyzed reaction product. To enhance fiber staining, nickel ammonium sulfate was added to the last step.

2.7. Cell counts and quantification of fiber outgrowth

Cell counts were made using light microscopy. Counts of TH+ cell bodies were made in every third section throughout the rostral-caudal extent of the lesioned/transplanted striatum. Particles less than 5.0 μm were not counted. The total number of TH+ cell bodies was summed for each animal and an average value (±S.E.M.) was calculated for each of the three different treatment groups. Cell counts were made with the observer blind to the treatment.

Fiber outgrowth from transplants was quantified using methodology from a previous study [35]. Briefly, low power (2×) images of brain sections containing TH immunostained transplants were captured via a video frame grabber and stored to computer disk as TIFF files; approximately six to eight brain sections containing grafts were used for analyses. Image files were analyzed on a Macintosh IIsi computer using the public domain NIH Image program. Coarse fibers, cell bodies, and fine granules immunostained for TH were distinguished from one another by their detection at different density levels. For example, fine TH-ir elements distributed diffusely within the host striatum were measured by adjusting density levels to exclude TH+ cell bodies and background from the calculation. All density measurements were made with the observer blind to the treatment.

2.8. Statistical analysis

Analysis of variance (ANOVA) was used to analyze the effect of treatment (transplantation time relative to lesion) on the dependent variables: rotational scores, cell counts, and area of fiber outgrowth. Results for the ELISA analysis were analyzed using ANOVA. Student-Newman-Keuls was used for post hoc mean comparisons for all ANOVAs showing a significant treatment effect. The alpha level was set to 0.05.

3. Results

3.1. Post-lesion measurements of BDNF or GDNF protein in striatum or ventral midbrain

Table 1 summarizes BDNF and GDNF protein in the striatum or ventral midbrain immediately after (3 days) or 12 weeks after naïve rats received unilateral 6-OHDA lesions. Levels of BDNF and GDNF protein are greater in the lesioned ventral midbrain than in the intact side 3 days after a 6-OHDA lesion. At this same time point, we do not

Table 1
Measurement of BDNF or GDNF 3 days or 12 weeks post-lesion (ng/g tissue)

Brain region	BDNF		GDNF	
	3 days	12 weeks	3 days	12 weeks
Striatum				
Intact side	11.1±0.8	11.5±0.6	9.0±0.4	11.1±0.4
Lesioned side	10.1±0.9	11.1±1.0	8.8±0.6	14.3±1.4
Ventral midbrain				
Intact side	10.8±0.6	13.0±1.0	9.1±0.3	10.7±0.1^
Lesioned side	16.0±1.5*	15.7±1.5	11.0±0.7^^	8.7±1.0

*P=0.007 versus intact side. ^P=0.06 versus lesioned side (approached significance). ^^P=0.02 versus intact side.

Table 2
Relative changes of BDNF or GDNF at several post-lesion time points

	3 Days	2 Weeks*	4 Weeks^	12 Weeks
BDNF				
Lesioned striatum	n.d.	↑	↑	n.d.
Lesioned ventral midbrain	↑	↑	↑	n.d.
GDNF				
Lesioned striatum	n.d.	↑	n.r.	n.d.
Lesioned ventral midbrain	↑	n.d.	n.r.	↓

↑, significant increase relative to intact side. ↓, significant decrease relative to intact side. n.d., no difference. n.r., not reported. * Data initially reported in [33]; ^ data initially reported in [32].

observe significant differences in protein levels between the lesioned and intact striatum for either BDNF or GDNF. At 12 weeks post-lesion, BDNF and GDNF protein levels are the same in the intact and lesioned sides for both the striatum and ventral midbrain. The mean value of GDNF protein in the intact ventral midbrain is greater than that in lesioned ventral midbrain, however, the statistical comparison of GDNF of these two means only approaches significance ($P=0.06$). Table 2 summarizes changes in the expression of BDNF and GDNF protein levels in the nigrostriatal pathway at various post-lesion time points.

3.2. Rotational behavior

Statistical analysis of rotational scores revealed a significant effect of treatment ($F(4,36)=2.92$, $P=0.03$). In Fig. 1, lesioned animals receiving transplants in all five treatment groups show functional compensation as determined by the low rates of amphetamine-induced rotational behavior observed in these animals 4 weeks after grafting. Statistical comparison of rotational scores revealed significantly lower scores for the 1 Week group when compared to the −1 Week group.

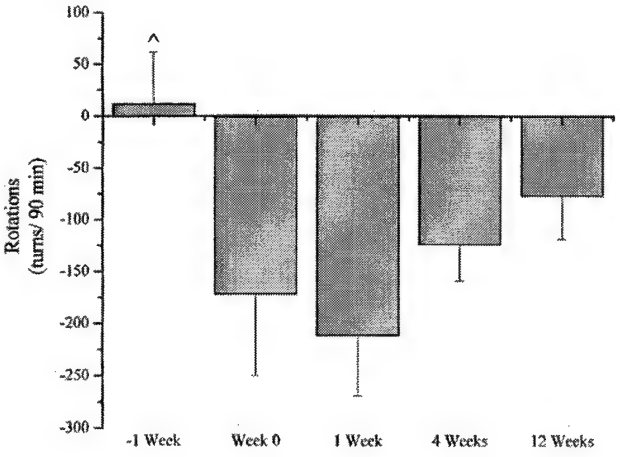

Fig. 1. Amphetamine-induced rotational scores for animals in each treatment group 4 weeks after grafting. Grafts were implanted at the following time points relative to the 6-OHDA lesion: 1 week before ($n=8$, −1 Week), at the same time, ($n=6$, Week 0), 1 week after ($n=9$, 1 Week), 4 weeks after ($n=8$, 4 Weeks), or 12 weeks after ($n=6$, 12 Weeks). Bars represent the average rotational score for each treatment group±S.E.M. Rotational behavior was induced with amphetamine (5.0 mg/kg, i.p.) and the total number of ipsilateral (positive) and contralateral (negative) rotations were counted over a 90-min post-injection period. Scores for the 1 Week group were significantly greater than the scores for −1 Week group. ×$P<0.05$, 1 Week versus −1 Week.

3.3. Cell counts of transplanted TH+ neurons

Statistical analysis of cell count data revealed a significant effect of treatment ($F(4,36)=2.67$, $P=0.05$). The average number of TH+ neurons in transplants for the 1 Week group was more than double and statistically greater than the average number counted in the −1 Week group (Fig. 2).

3.4. Fiber outgrowth

Statistical analysis of fiber outgrowth revealed a significant effect of treatment ($F(4,36)=3.30$, $P=0.02$). Similar to the cell count data, animals in the 1 Week group showed an average area of TH+ fiber staining in the host tissue surrounding the transplant that was over double the area of TH+ fiber staining observed in the −1 Week group (Fig. 3). The area of TH+ fiber outgrowth was significantly greater for animals in the Week 0 or 4 Weeks groups than in the −1 Week group. Fig. 4 shows the four best examples of TH+ fiber staining in the lesioned/trans-

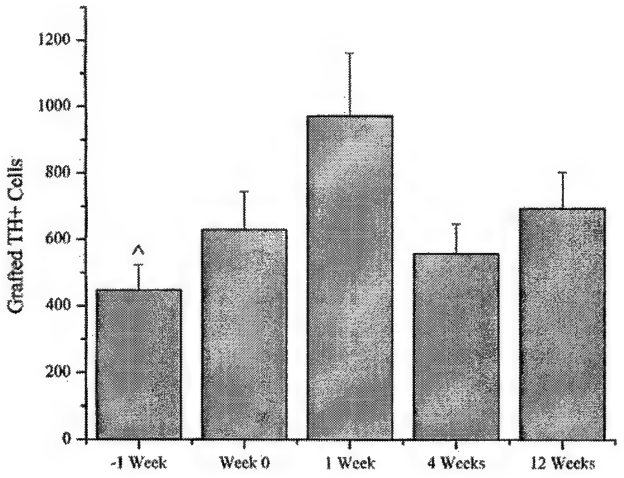

Fig. 2. Total number of TH+ cell bodies counted in dopamine grafts for each of the five treatment groups. Grafts were implanted at the following time points relative to the 6-OHDA lesion: 1 week before ($n=8$, −1 Week), at the same time, ($n=6$, Week 0), 1 week after ($n=9$, 1 Week), 4 weeks after ($n=8$, 4 Weeks), or 12 weeks after ($n=6$, 12 Weeks). Brains were sliced into 40-μm sections and immunohistochemically stained for TH. The total number of TH+ cell bodies was counted in every third section throughout the rostral-caudal extent of the lesioned/transplanted striatum. Bars represent an average of the total number of TH+ cell bodies for each animal in each treatment group±S.E.M. Cell counts for the 1 Week group were significantly greater than cell counts for the −1 Week group. ×$P<0.05$, 1 Week versus −1 Week.

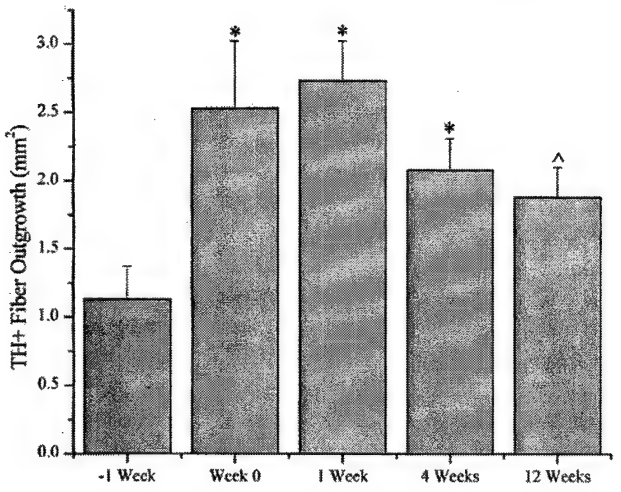

Fig. 3. Total area of TH+ fiber outgrowth from dopamine grafts for each treatment group 5 weeks after grafting. Grafts were implanted at the following time points relative to the 6-OHDA lesion: 1 week before (n=8, −1 Week), at the same time, (n=6, Week 0), 1 week after (n=9, 1 Week), 4 weeks after (n=8, 4 Weeks), or 12 weeks after (n=6, 12 Weeks). Brains were sliced into 40-μm sections and immunohistochemically stained for TH. Densitometry was set to detect TH+ fibers projecting from the graft and TH+ reinnervation of the host striatum; TH+ cell bodies and fibers within the transplant, as well as background, were excluded from the analysis. Area calculations were made in every third section throughout the rostral-caudal extent of the lesioned/transplanted striatum and an average area of TH+ fiber outgrowth was calculated for each animal. Bars represent an average area of fiber outgrowth for each treatment group±S.E.M. Fiber outgrowth for the 1 Week group was significantly greater than outgrowth for the −1 Week or 12 Week groups. *P<0.05 versus −1 Week, ×P<0.05 versus Week 1.

planted and intact striata for both the −1 Week and 1 Week groups.

3.5. Correlation of behavior and graft morphology

The behavioral and morphological scores presented above were pooled for all treatment groups and individual scores for rotational behavior were plotted as a function of the number of TH+ neurons in the graft (Fig. 5A) or as a function of the area of TH+ outgrowth from the graft (Fig. 5B). Regression analysis was performed on these scatter plots and our analysis revealed that the decrease in rotational scores following grafting is more tightly correlated with the extent of grafted fiber outgrowth (r^2=−0.71) than it is with the number of TH+ neurons within the graft (r^2=−0.35).

4. Discussion

The results of this study show that factors within the denervated striatum provide a more enriched environment than the intact striatum for graft development and function. Grafts placed into the denervated striatum within a 1-month period after the nigrostriatal pathway is lesioned show significantly better fiber outgrowth than grafts placed initially into an intact striatum. The survival of grafted dopamine neurons is also improved if the grafts are placed into the denervated striatum within 1 week after the nigrostriatal pathway lesion. These results suggest that in young rats, a neurotoxic lesion of the nigrostriatal pathway induces an increase of neurotrophic activity that is beneficial to the survival and functional outgrowth of fetal dopamine grafts. Moreover, this effect may be transient and dependent upon the length of time between the lesion and grafting procedures.

It is interesting that fiber outgrowth from fetal dopamine grafts is improved at the same post-lesion time points when specific dopaminergic neurotrophic factors are known to increase their expression in the lesioned striatum relative to the intact striatum. In previous studies we observed an improvement of fiber outgrowth from dopamine grafts when grafts were exposed to continuous infusion of exogenous BDNF during the 1st month after grafting [35] or for a 2-week infusion period that began at the end of the 2nd post-transplantation week [34]; Sauer et al. reported that BDNF infusions into dopamine grafts improve function without a concomitant increase in the number of surviving grafted dopamine neurons [27]. In young adult rats, we and others observe an increase in BDNF protein levels within the lesioned striatum that is apparent 2–4 weeks after the lesion [32,33,39]; not only is striatal BDNF elevated 2 weeks after a nigrostriatal pathway lesion, but so is another dopaminergic neurotrophic factor, GDNF. In this study we also measured BDNF and GDNF protein in animals with lesions only and did not observe an increase of either neurotrophic factor in the lesioned striatum immediately (3 days) or 12 weeks after the lesion. This finding, combined with the results from our earlier studies, suggests that neurotoxic lesions induce transient increases in neurotrophic factor expression in the striatum for a period of at least 1 month that may not begin immediately after the administration of the neurotoxin. Of all the treatment groups tested in this study, dopamine grafts implanted into the 1 Week group would most likely be exposed to elevated levels of endogenous BDNF and GDNF at the same time period when exogenous infusion of these neurotrophic factors improves the survival and fiber outgrowth of fetal dopamine grafts. The transient increase of neurotrophic factors within the lesioned striatum may be one explanation why the area of TH+ fiber outgrowth from grafts is higher when the grafts are implanted post-lesion rather than pre-lesion. Nonetheless, it is clear that removal of dopaminergic afferents to the striatum is a requirement to stimulate significant fiber outgrowth from fetal dopamine grafts.

The relatively poor fiber outgrowth and survival of grafted dopamine neurons observed in the −1 Week group could be attributable to several factors. While a lack of lesion-induced increase of neurotrophic activity may be one likely explanation for diminished fiber outgrowth, it is

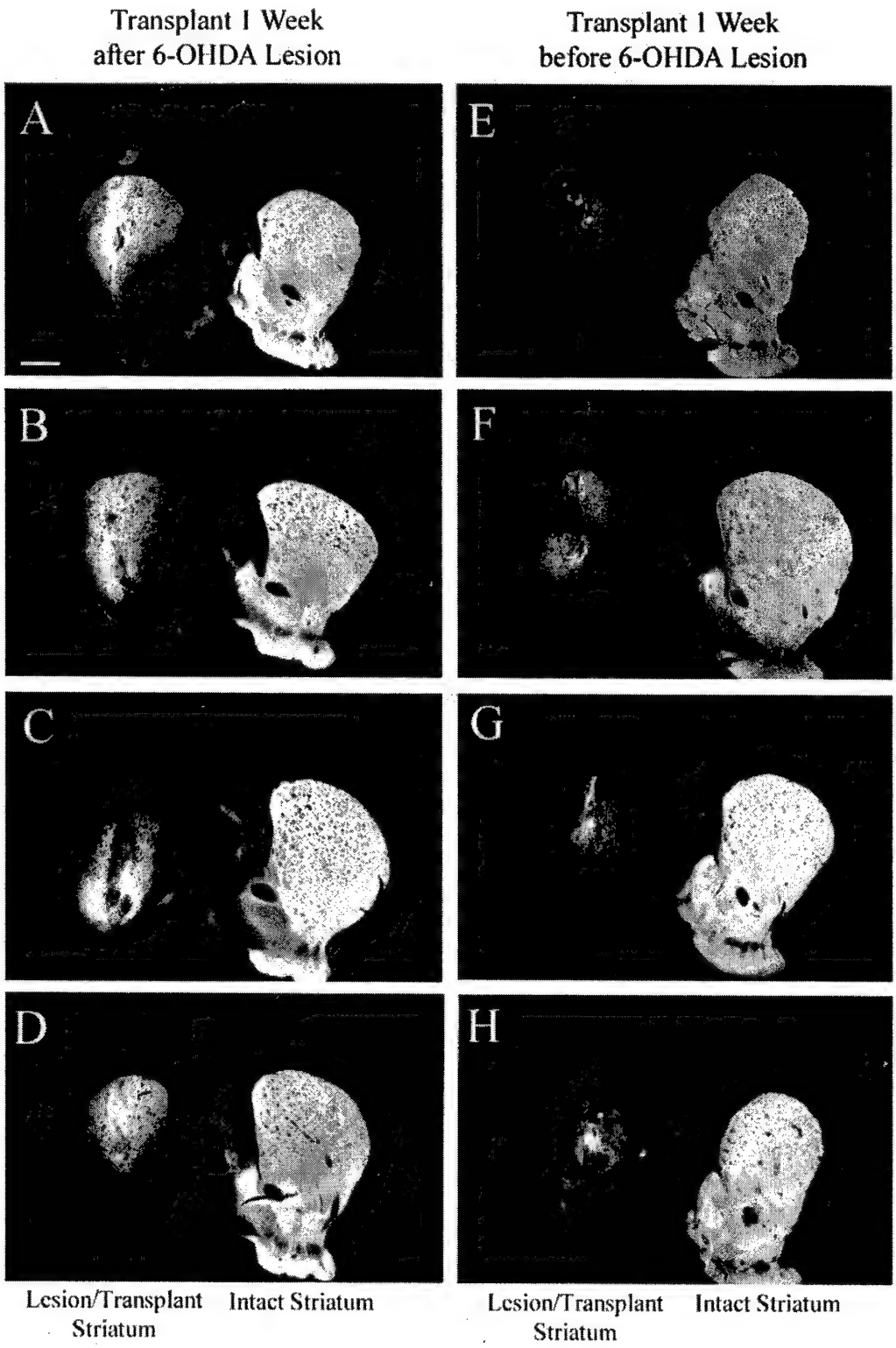

Fig. 4. Dark-field photomicrographs of coronal brain sections stained for TH and showing the four best examples of TH+ staining in the lesioned/transplanted striatum for the 1 Week (left column, panels A–D) and −1 Week (right column, panels E–H) treatment groups. For each panel, the left side of the brain is the lesioned/transplanted side and the right side is the intact side. Each panel is from a different animal. Note the larger area of TH+ fiber outgrowth in lesioned/transplanted striatum of the 1 Week group when compared to same region in the −1 Week group. Brain sections are 40 μm in thickness. Calibration bar in panel A: 1 mm.

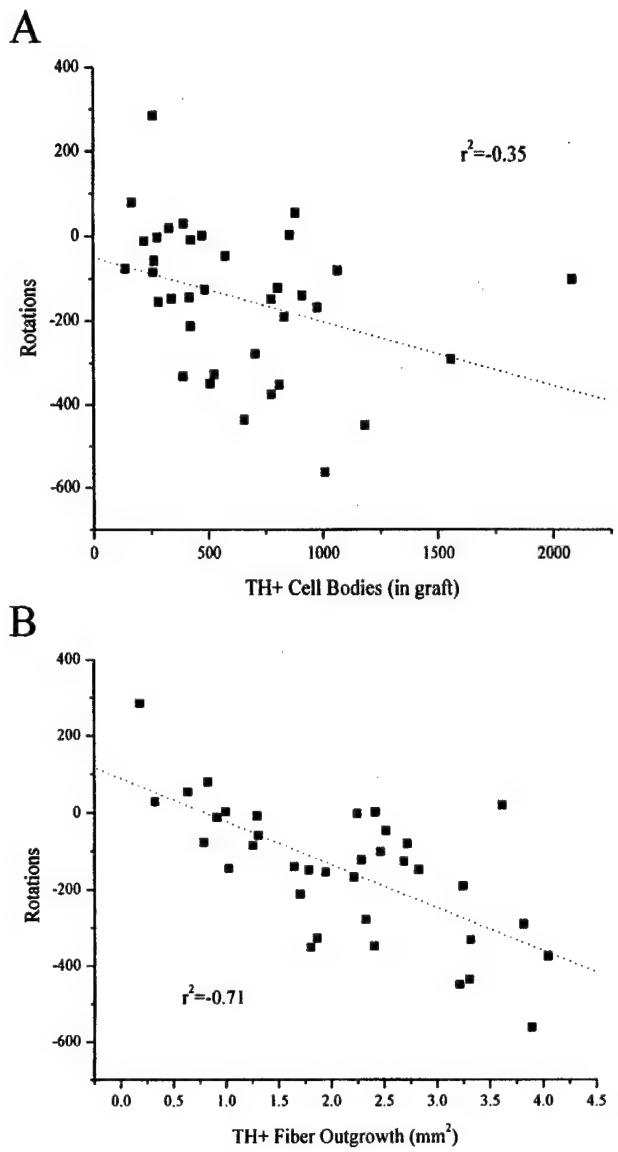

Fig. 5. Correlations between rotational scores, the number of grafted TH+ neurons, and TH+ fiber outgrowth. Scatter plots of individual rotational scores (n=37) were plotted as a function of either the number of TH+ neurons within the animal's graft (A) or the area of TH+ fiber outgrowth from the animal's graft (B). A linear regression analysis was performed on each scatter plot (dotted line). The correlation coefficients for plots (A) and (B) are $r^2=-0.35$ and $r^2=-0.71$, respectively. The reversal of amphetamine-induced rotational behavior by dopamine grafts is more tightly correlated to the area of fiber outgrowth than it is to the number of grafted TH+ neurons.

certainly not the only explanation. For example, grafts implanted into the intact striatum would have to compete with existing dopamine fibers in order to establish functional contacts with target neurons in the host tissue. The limited fiber outgrowth from transplants placed into the intact striatum may be restricted to sites where host dopaminergic neurons are disrupted during the implantation process. A previous study also reported anecdotally that fetal dopamine grafts placed into the intact striatum had a more restricted fiber outgrowth pattern than grafts placed into the denervated striatum [16]. Also, the lower survival rate of TH+ cells within the transplants of the −1 Week group may be directly related to the inability of transplanted neurons to successfully innervate the host tissue. During normal development of the nigrostriatal pathway, midbrain dopamine neurons undergo several stages of programmed cell death that might be a consequence of many immature neurons competing to establish functional contacts with a limited number of targets [19]. Moreover, implanting immature dopamine neurons into a dopamine-rich environment may be detrimental to their survival based upon evidence that dopamine itself may induce apoptosis in developing neurons [40]. Therefore the decreased survival of transplanted neurons in the −1 Week group cannot be entirely explained by the neurotrophic environment of the host brain at the site of implantation. Indeed, results from studies performed in our laboratory and others have shown that the intact striatum maintains expression of several dopaminergic neurotrophic factors in adult brain [33,36,39].

While the area of TH+ fiber outgrowth from grafts was significantly greater for the Week 0 or 4 Weeks groups when compared to the −1 Week group, we observed that the number of TH+ neurons in grafts for the Week 0 or 4 Weeks group was slightly higher but not significantly greater than the number of TH+ neurons in grafts for the −1 Week group. On the other hand, both the number of TH+ neurons and the area of fiber outgrowth were significantly greater for the 1 Week group when statistically compared to the −1 Week group. This suggests that a dynamic change in the neurotrophic environment of the denervated striatum may be occurring during the 1st month after the lesion and/or the 1st month after grafting. Our previous studies have demonstrated that after 1–2 weeks following a 6-OHDA lesion, both survival and outgrowth factors may be up-regulated in the denervated striatum and thus provide an environment that supports the survival and functional outgrowth of grafted neurons. At 4 weeks after the lesion, however, survival factors within the denervated striatum may decline whereas outgrowth factors remain elevated. Interestingly, we observe in young lesioned rats an elevation of BDNF levels in the denervated striatum 4 weeks after a nigrostriatal pathway lesion [32]. As already mentioned, BDNF may have properties of a target-derived neurotrophic factor that stimulates fiber outgrowth more than it does as a survival factor for fetal dopamine grafts. Interestingly, fiber outgrowth in the 12 Weeks group is significantly less than that observed in the 1 Week group, and this corresponds to the same post-lesion period when striatal BDNF is not significantly elevated in the striatum of rats with lesions only. Likewise, we observe that GDNF is significantly elevated in the denervated striatum 2 weeks after a 6-OHDA lesion, but this elevation may only be transient because we do not observe a significant differ-

ence in GDNF protein between the intact and lesioned striatum during the 12th post-lesion week. Glial cell line-derived neurotrophic factor is known to be a potent survival factor for dopamine neurons in vitro and in vivo [1,4,8,11,13,21–23,26,28,31]. If striatal GDNF levels are increased only transiently during the first 2 weeks following a 6-OHDA lesion, then this may provide a partial explanation why the number of TH+ neurons was significantly greater in the 1 Week group than in the −1 Week group, and why the comparison of TH+ neurons for the −1 Week, Week 0, and 4 Weeks groups did not yield a significant difference.

We also observe that graft-mediated reduction of amphetamine-induced rotational behavior was more tightly correlated with the degree of fiber outgrowth from the graft than it was with the actual number of surviving TH+ neurons within the graft. Grafted rats showing the largest areas of fiber outgrowth also showed the highest degree of functional overcompensation when tested with amphetamine. The phenomenon of overcompensation in amphetamine-induced rotational behavioral has been ascribed to grafted fibers forming contacts with corticostriatal fibers because the abolition of corticostriatal afferents also blocks this over-compensatory response [6]. Another explanation for this over-compensatory response to amphetamine may be related to the status of striatal dopamine receptors or to an inefficient reuptake of dopamine by grafted neurons; it still remains uncertain whether striatal dopamine receptors are fully normalized by the grafts. From a therapeutic standpoint, it remains to be determined whether or not fiber contacts made between the graft and host neurons are aberrant or functionally useful. Nevertheless, the extent of fiber outgrowth from grafts may be a better predictor of graft-mediated reduction of amphetamine-induced rotational behavior than the actual number of surviving grafted TH+ neurons. This is consistent with the earlier report that behavioral recovery is correlated with the extent of graft fiber reinnervation of the host brain [2,10,29].

It would be interesting to observe whether or not dopamine grafts show enhanced survival and function in the lesioned striatum of aged rats. Studies from our laboratory indicate that protein levels of at least two neurotrophic factors, BDNF and GDNF, are greater in lesioned striatum than in the intact striatum in young rats whereas there are no significant differences in either BDNF or GDNF protein levels between the lesioned and intact striata of aged rats [32,33]. The results of these studies suggest that the neurotrophic environment of the denervated striatum of aged rats may be comparable to the intact striatum of young or old rats. This is intriguing because Collier et al. [9] recently reported that dopamine grafts showing improved transplant function in young animals were virtually without effect in aged rats; this study also reported impaired morphological development of grafts and, in particular, a reduction of fiber outgrowth from grafts placed into aged denervated striatum. In the present study we observed a significant reduction of fiber outgrowth from grafts placed into the intact striatum. The significance of these studies may be more fully appreciated in light of the results from a recent clinical trial using dopamine neuron implants in Parkinson's patients that concluded that patients under 60 years of age exhibited statistically significant clinical benefits from transplants while patients older than 60 years of age did not [15]. It is conceivable that while dopamine grafts placed into elderly Parkinson's patients show evidence of graft survival in terms of PET scan studies, the functional outgrowth of these grafts may be impaired due to an impoverished neurotrophic environment. This may be one explanation why younger Parkinson's patients benefit more from dopamine grafts than older patients.

In conclusion, neurotoxic lesions of the nigrostriatal pathway may induce a transient increase of neurotrophic activity that is initially beneficial to the survival and function of dopamine grafts. In addition, the length of time between the lesion and the grafting procedure may have a direct effect on the success of grafted fetal dopamine neurons in terms of their survival and functional reinnervation of the host. These effects may be directly related to the reports from other studies that have provided evidence that neurotoxic lesions of the nigrostriatal pathway induce a compensatory increase of neurotrophic activity in the denervated striatum.

Acknowledgements

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Appendix 4

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Striatal trophic factor activity in aging monkeys with unilateral MPTP-induced parkinsonism

Timothy J. Collier[a,*], Zao Dung Ling[b], Paul M. Carvey[b], Paul M. Carvey[b], Anita Fletcher-Turner[c], David M. Yurek[c], John R. Sladek Jr.[d], Jeffrey H. Kordower[a]

[a]*Department of Neurological Sciences, Rush University Medical Center, Chicago, IL 60612, USA*
[b]*Department of Pharmacology, Rush University Medical Center, Chicago, IL 60612, USA*
[c]*Department of Neurosurgery, University of Kentucky School of Medicine, Lexington, KY 40536, USA*
[d]*Department of Psychiatry, University of Colorado Health Sciences Center, Denver, CO 80262, USA*

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Abstract

Striatal trophic activity was assessed in female rhesus monkeys of advancing age rendered hemiparkinsonian by unilateral intracarotid administration of MPTP. Three age groups were analyzed: young adults (8–9.5 years) $n = 4$, middle-aged adults (15–17 years) $n = 4$, and aged adults (21–31 years) $n = 7$. Fresh frozen tissue punches of caudate nucleus and putamen were collected 3 months after MPTP treatment and assayed for combined soluble striatal trophic activity, brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF).

This time point was chosen in an effort to assess a relatively stable phase of the dopamine (DA)-depleted state that may model the condition of Parkinson's disease (PD) patients at the time of therapeutic intervention. Analyses were conducted on striatal tissue both contralateral (aging effects) and ipsilateral to the DA-depleting lesion (lesion × aging effects). We found that combined striatal trophic activity in the contralateral hemisphere increased significantly with aging. Activity from both middle-aged and aged animals was significantly elevated as compared to young adults. Following DA depletion, young animals significantly increased combined striatal trophic activity, but middle-aged and aged animals did not exhibit further increases in activity over their elevated baselines. BDNF levels in the contralateral hemisphere were significantly reduced in aged animals as compared to young and middle-aged subjects. With DA depletion, BDNF levels declined in young and middle-aged animals but did not change from the decreased baseline level in old animals. GDNF levels were unchanged with aging and at 3 months after DA depletion. The results are consistent with several conclusions. First, by middle age combined striatal trophic activity is elevated, potentially reflecting a compensatory reaction to ongoing degenerative changes in substantia nigra DA neurons. Second, in response to DA depletion, young animals were capable of generating a significant increase in trophic activity that was sustained for at least 3 months. This capacity was either saturated or was not sustained in middle-aged and aged animals. Third, the aging-related chronic increase in combined striatal trophic activity was not attributable to BDNF or GDNF as these molecules either decreased or did not change with aging.
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Keywords: Neurotrophic; Monkey; Nonhuman primate; GDNF; BDNF; Substantia nigra; Striatum; Dopamine; MPTP; Parkinsonism

Introduction

Animal models are essential tools for understanding neural mechanisms associated with neurodegenerative diseases and for the design of effective experimental therapeutic interventions. One primary risk factor for neurodegenerative disease is advancing age (Baldereschi et al., 2003; Lindsay et

* Corresponding author. Department of Neurological Sciences, Rush University Medical Center, Cohn Building, Suite 300, 1735 West Harrison Street, Chicago, IL 60612. Fax: +1 312 563 3571.
E-mail address: tcollier@rush.edu (T.J. Collier).

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al., 2002; Wakisaka et al., 2003). While there are several animal models of Parkinson's disease (PD) (Cenci et al., 2002; Collier et al., 2003a,b; Orth and Tabrizi, 2003; Shimohama et al., 2003), the impact of aging on the brain's response to dopamine (DA) depletion in these models has not received much attention until recently. Indeed, the common use of young adult animals depleted of striatal DA as a test system for novel therapies for PD may yield overly optimistic views of efficacy. It is unlikely that compensatory mechanisms expressed in the injured young adult brain remain fully functional in the aged brain, when individuals most often manifest neurodegenerative disease. In this regard, we have demonstrated that embryonic DA neurons grafted into aged hosts survive more poorly and exert a less potent therapeutic benefit than identical grafts placed in young hosts in a rat model of PD (Collier et al., 1999; Sortwell et al., 2001). These studies were predictive of data collected in a double-blind human clinical transplant trial in which superior benefit was observed in younger patients than in older patients (Freed et al., 2001). We have identified one potential contributing factor to the poorer graft outcome in aged rats: an aging-related reduction in striatal DA neurotrophic activity (Kaseloo et al., 1996; Ling et al., 2000). Indeed, supplementation of trophic support in the environment of grafted DA neurons dramatically enhances graft survival in aged rat hosts (Collier et al., 1999).

The present study sought to determine whether the development of an impoverished striatal neurotrophic environment resulting from advancing age presents a challenge for experimental therapeutics in a species more closely related to humans. We compared measures of striatal trophic factors for DA neurons in the intact and DA-depleted hemispheres of female rhesus monkeys of advancing age treated with unilateral intracarotid administration of MPTP. The striatum contralateral to MPTP lesion permits assessment of trophic factors as affected primarily by aging processes. The striatum ipsilateral to MPTP lesion allows for the assessment of these molecules in the context of the interaction between aging and severe DA depletion. Samples from the DA-depleted hemisphere were collected at 3 months after MPTP treatment. This time point was chosen in an effort to assess a relatively stable phase of the DA-depleted state that may model the condition of PD patients embarking upon a therapeutic intervention. Three assays were conducted. First, we assessed the ability of aggregate soluble trophic activity in extracts of the striatum to support DA neurons in culture. Trophic activity measured in this fashion is inversely related to DA tone in preclinical studies (Carvey et al., 1989, 1991, 1993a,b, 1996; Nijima et al., 1990; Tomozawa and Appel, 1986) and is increased in PD (Carvey et al., 1993; Yu et al., 1994). Second, brain-derived neurotrophic factor (BDNF) levels were assayed by enzyme-linked immunosorbent assay (ELISA). Converging lines of evidence support the concept that BDNF is a potent trophic factor for DA neurons in vitro and in vivo (Collier and Sortwell, 1999). BDNF is present in substantia nigra DA neurons (Seroogy and Gall, 1993; Seroogy et al., 1994) and in the striatum (Conner et al., 1997; Kawamoto et al., 1996) is regulated during nigral development (Friedman et al., 1991), increases in response to DA depletion (Funa et al., 1996; Yurek and Fletcher-Turner, 2000, 2001; Zhao et al., 1996), and the response to DA depletion is lost with aging in rats (Yurek and Fletcher-Turner, 2000, 2001). Substantia nigra BDNF is decreased in PD (Howells et al., 2000; Mogi et al., 1999; Parain et al., 1999). Third, glial cell line-derived neurotrophic factor (GDNF) was assayed by ELISA. GDNF also is a potent trophic factor for DA neurons in vitro and in vivo (Collier and Sortwell, 1999). GDNF is retrogradely transported from the striatum to the substantia nigra (Ai et al., 2003; Kordower et al., 2000; Wang et al., 2002). Within the nigra, GDNF is found in astrocytes (Choi-Lundberg and Bohn, 1995; Schaar et al., 1993), is regulated during development (Choi-Lundberg and Bohn, 1995; Stromberg et al., 1993), and declines with aging in rats (Yurek and Fletcher-Turner, 2001). Immunohistochemical studies have reported decreases in nigral GDNF in PD (Chauhan et al., 2001).

Our findings indicate that total trophic activity is significantly increased in the intact striatum by middle age and remains elevated in old age. In response to DA depletion, this combined activity is significantly increased in young adult monkeys but is not elevated above the already increased basal levels in middle-aged and aged monkeys. Striatal BDNF levels decrease significantly in the intact striatum in the oldest age group only and following DA depletion in young and middle-aged animals declines to the same extent seen in aged monkey. No significant changes were detected in striatal GDNF levels with aging or in response to DA depletion at 3 months post-MPTP.

Materials and methods

Animals

Subjects were female rhesus monkeys (*Macaca mulatta*) weighing 6–9 kg. Three age groups were studied: young adult (8–9.5 years) $n = 4$, middle-aged (15–17 years) $n = 4$, and aged (21–31 years) $n = 7$. Rhesus monkeys age at a rate of 3:1 as compared to humans (Andersen et al., 1999). Thus, our groups model the equivalent of 24 years, 45–51 years, and 63–93 years of human life. Animals were housed in individual primate cages and cared for in the AALAC approved Biological Resources Laboratory at the University of Illinois-Chicago. All monkeys were treated with unilateral intracarotid administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (3–4 mg) as previously described (Emborg et al., 2001). Treatment resulted in equivalent behavioral signs in all subjects, principally characterized by complete disuse of the forelimb contralateral to infusion. Care and use of these animals was in

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compliance with all applicable laws and regulations as well as principles expressed in the National Institutes of Health, United States Public Health Service Guide for the Care and Use of Laboratory Animals. This study was approved by the Animal Care and Use Committees of the University of Illinois-Chicago and Rush University Medical Center.

Tissue

Three months following induction of behavioral symptoms, animals were killed by pentobarbital overdose (50 mg/kg with effect confirmed by absence of corneal reflex) and perfused with physiological saline. Brains were removed, and each forebrain was divided into coronal slabs of 4 mm thickness on ice. Tissue punches, 1.3 mm in diameter, were taken from standard locations (Sladek et al., 1995) in the ventral–medial caudate nucleus and putamen at precommissural and commissural levels of the striatum. Punches were frozen on dry ice and stored at −70°C until processing. Caudate and putamen punches from the precommissural striatum were devoted to the assay of combined soluble trophic activity, and punches from the commissural striatum were devoted to ELISAs for BDNF and GDNF. Samples from all animals were not available for all assays. For all assays, measures derived from caudate nucleus and putamen were not statistically different and were combined for presentation as "striatal" trophic factor activity or levels.

Trophic activity and tissue culture

Striatal tissue was homogenized in ice-cold Hank's balanced salt solution (HBSS), centrifuged at 18,000 × *g* for 15 min, and the protein concentration of the supernatant extracts was assessed for total protein using the Bio-Rad kit. The extract was adjusted to 200 μg protein/ml and assessed for trophic activity as described previously (Ling et al., 2000). Briefly, ventral mesencephalon from embryonic day 14.5 rats was dissected and dissociated into a cell suspension. Cells were plated at 125,000 cells/cm 2 in 96-well plates and incubated in 75% serum-free-defined media (DM) + 25% striatal tissue extract. On every plate, controls were used to assess baseline growth. These control cultures were incubated in 75% DM + 25% HBSS instead of striatal extract. Each plate also had 2 wells in which cells were incubated in 90% DM + 10% fetal calf serum. These positive controls were used to establish that cultures were growth responsive. Plates in which the tyrosine hydroxylase immunoreactive (THir) cell counts in the positive controls were not at least 2× that seen in plate controls were discarded. After incubation for 72 h, cultures were fixed, stained, and counts of THir neurons were performed. Cells were counted in a cross pattern covering 44% of the well surface. The THir cell counts in each well were divided by the average THir cell count in the plate controls and used as a survival index. Each extract was tested in at least two independent culture runs.

Enzyme-linked immunosorbent assay (ELISA) for BDNF and GDNF

ELISAs for BDNF and GDNF followed previously published protocols (Yurek and Fletcher-Turner, 2000, 2001). Each tissue sample was weighed prior to freezing at −80°C. Subsequently, tissue samples were homogenized in 25 volumes of buffer [400 mM NaCl, 0.1% Triton-X, 2.0 mM EDTA, 0.1 mM benzethonium chloride, 2.0 mM benzamidine, 0.1 mM PMSF, Aprotinin (9.7 TIU/ml), 0.5% BSA, 0.1 M phosphate buffer, pH 7.4]. The homogenate was centrifuged for 10 min at 10,000 × *g* at 4°C. The neurotrophic factor content was determined in 100 μl aliquots of supernatant with an antibody sandwich format: extracted neurotrophic factor from each sample was captured with a neurotrophic factor-specific monoclonal antibody (mAb), the captured neurotrophic factor was then bound to the second, neurotrophic factor-specific polyclonal antibody (pAb). After washing, the amount of specifically bound pAb was detected using a species-specific anti-IgY antibody conjugated to horseradish peroxidase (HRP) as a tertiary reactant. Unbound conjugate was removed by washing, and following an incubation period with a chromogenic substrate the color change was measured. The amount of BDNF or GDNF is proportional to the color change generated in an oxidation-reduction reaction (Promega E_{max}™ Immuno-Assay System) and detected in a microplate reader set at 450 nm.

Statistics

Comparisons of counts of THir neurons in culture and trophic factor levels were analyzed with analysis of variance (ANOVA) followed by Fisher's protected least significant differences (PLSD) test.

Results

Combined striatal trophic activity

There was an aging-related increase in the combination of soluble trophic factors derived from the caudate nucleus and putamen contralateral to MPTP exposure. This was demonstrated by the significant increase in the capacity for this trophic activity to support survival and growth of cultured rat DA neurons [$F(5,49) = 3.857$, $P = 0.005$]. The approximate 50% increase in survival of THir neurons in culture was evident by middle age and sustained in old age (Figs. 1 and 2). For young adult monkeys, extracts derived from the DA-depleted hemisphere produced a significant 50% increase in survival of cultured DA neurons, matching the elevated baseline levels of older monkeys. In contrast, samples from the DA-depleted hemisphere of middle-aged and aged subjects maintained

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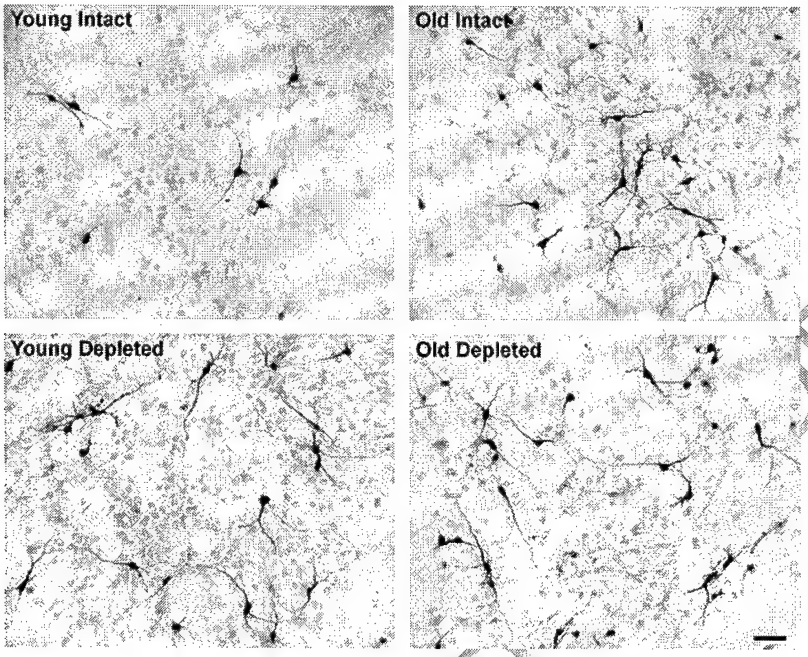

Fig. 1. Striatal-derived trophic activity for cultured rat dopamine (DA) neurons is increased with aging and in response to DA depletion in young adult, but not aged, monkeys. Micrographs illustrate representative fields of cultured embryonic day 14.5 rat ventral mesencephalon immunostained for tyrosine hydroxylase (TH) to visualize DA neurons. Cultures were exposed to striatal extracts from monkeys of varying ages for 72 h and quantified for TH-positive cell numbers relative to control cultures not exposed to extracts. As shown, extracts from the intact striatum exhibit an aging-related increase in trophic support for DA neurons. Comparison of effects of extracts from the intact and DA depleted hemispheres of young and old monkeys indicates that striatal DA depletion triggers increased trophic activity in young adult monkeys, but that aged monkeys do not exhibit any further increase in trophic activity over their already elevated baseline levels. Scale bar = 50 μm.

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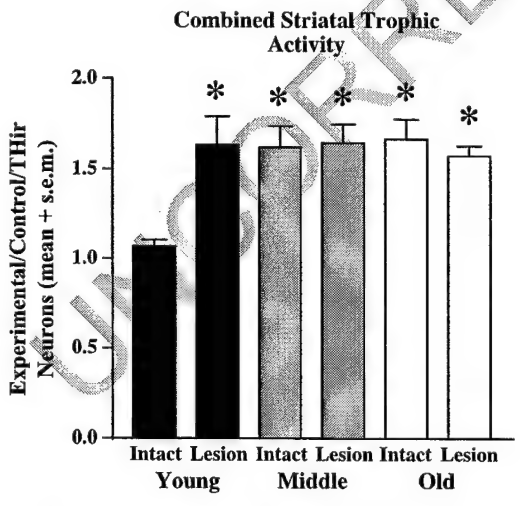

Fig. 2. Combined nonhuman primate striatal trophic activity for cultured dopamine (DA) neurons. Counts of tyrosine hydroxylase (TH)-positive neurons in cultures exposed to striatal extracts are presented as compared to control cultures not exposed to extracts. For extracts derived from the intact striatum, an approximately 50% increase in trophic activity is detectable by middle age and sustained in old age. In response to striatal DA depletion, young adult monkeys generate a similar 50% increase in trophic activity, but further increases beyond elevated baseline levels are not produced in middle-aged and aged monkeys. ANOVA: $F(5,49) = 3.857$, $P = 0.005$. Fisher's PLSD: $^{*}P < 0.002$ for young intact as compared to all other groups.

increased trophic support but were not elevated above the already increased levels of the contralateral hemisphere attributable to aging.

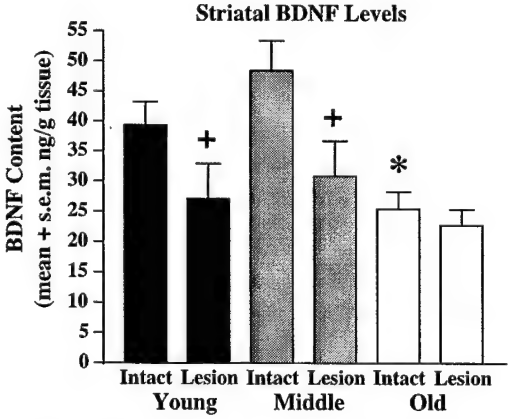

Fig. 3. Striatal BDNF levels as measured with ELISA. BDNF levels are presented as nanograms of BDNF per gram tissue. For the intact striatum, BDNF levels were found to be maintained from young adulthood into middle age but decreased significantly in old age. Following dopamine depletion, young adult and middle-aged monkeys exhibited significant decreases in striatal BDNF. Aged monkeys showed no further decline in BDNF beyond their depleted baseline levels. ANOVA: $F(5,42) = 4.508$, $P = 0.002$. Fisher's PLSD: $^{+}P < 0.05$ as compared to intact hemisphere; $^{*}P < 0.02$ as compared to intact hemisphere of young and middle-aged monkeys.

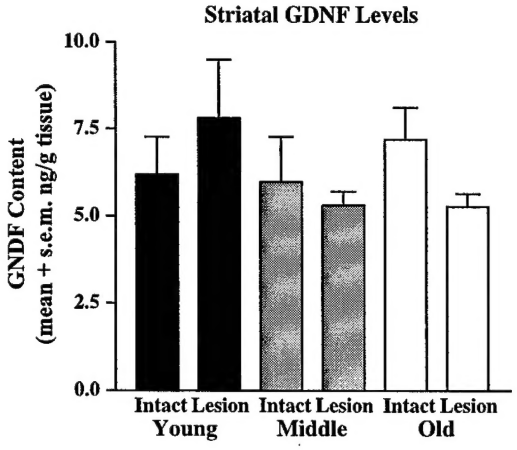

Fig. 4. Striatal GDNF levels as measured with ELISA. GDNF levels are presented as nanograms of GDNF per gram tissue. No differences were found in striatal levels of GDNF with aging or at 3 months after dopamine depletion. ANOVA: $F(5,38) = 0.729$, $P = 0.606$.

Striatal BDNF levels

BDNF levels measured in striatal samples from the hemisphere contralateral to MPTP exposure exhibited a significant aging-related decline of approximately 40% that was detectable only in the oldest age group [$F(5,42) = 4.508$, $P = 0.002$] (Fig. 3). In response to DA depletion, striatal BDNF decreased significantly in young adult and middle-aged subjects but did not decrease further in the already BDNF-depleted-aged subjects.

Striatal GDNF levels

GDNF levels assayed from the striatum of the hemisphere contralateral to MPTP exposure showed no changes associated with advancing age [$F(5,38) = 0.729$, $P = 0.606$] (Fig. 4). Similarly, at 3 months after MPTP-induced DA depletion, striatal GDNF levels were not significantly different from levels in the contralateral hemisphere.

Discussion

The expression of neurotrophic factors is a dynamic process and a defining event associated with nervous system development, aging, and the response to damage and disease. For the nigrostriatal system, BDNF and GDNF perhaps are the most studied of the more than 20 molecules with known neurotrophic effects for DA neurons (Collier and Sortwell, 1999). Abundant information exists in the literature on the regulation of neurotrophic factors during early development of the nervous system, and these data serve in part as the rationale for their potential therapeutic efficacy. Our understanding of how aging influences neurotrophic function is in its relatively early stages. Most studies examining aging effects of trophic factors upon the nigrostriatal DA system have been conducted on aged rats. Virtually no such studies have been performed in aging nonhuman primates, and the data collected in our study present several differences from what has been found in rodents.

Similar to the present study, the majority of evidence on striatal trophic factor levels obtained from aging rats used a unilateral DA depletion model. The hemisphere contralateral to unilateral infusion of 6-hydroxydopamine (6-OHDA) has been assayed for changes in trophic factors as a function of chronological age and the hemisphere ipsilateral to 6-OHDA was assayed for the reaction of the aging striatum to severe DA depletion. It should be noted that the use of the hemisphere contralateral to unilateral DA depletion might not be a perfect representation of a truly "intact" striatum from untreated animals. Unilateral lesions will remove the very small percentage of nigrostriatal DA fibers that cross to the contralateral striatum (Hedreen and DeLong, 1991), and in the case of intracarotid administration of MPTP there can be evidence of exposure contralaterally (Eberling et al., 2002). The presence or absence of crossover effects appears to be dose related, occurring with increased frequency at relatively higher doses (Guttman et al., 1990). For the present study, parallel analysis of substantia nigra cell numbers in the same monkeys using unbiased stereology has found no difference between counts in the hemisphere contralateral to MPTP exposure and counts derived from untreated monkeys. Thus, for the cases presented here, we have no evidence of significant MPTP exposure to the contralateral hemisphere. With this potential caveat in mind, we will refer to the hemisphere contralateral to unilateral DA depletion as "intact," for convenience, in the remainder of the discussion.

Studies in aging rats have been conducted using the same assays reported here. For effects on combined striatal trophic activity attributable to aging, parallel analysis of the striatum of untreated rats and the striatum contralateral to a unilateral 6-OHDA lesion detected identical results. This supports the view that the hemisphere contralateral to unilateral DA depletion can be appropriate for study as an intact control. For aging rats, combined striatal trophic activity for cultured DA neurons derived from intact striatum was relatively preserved through middle age (4, 12, and 18 months old) but declined significantly in advanced age (23 months old) (Ling et al., 2000). Striatal levels of GDNF, but not BDNF, decreased significantly in aged rats (4–5 months old as compared to 31–34 months old) (Yurek and Fletcher-Turner, 2000, 2001).

Our evidence from the intact hemisphere of aging monkeys is considerably different from the pattern detected in rats. We found that combined striatal trophic activity for cultured DA neurons is significantly increased by middle age and remains elevated in old age. No change in striatal GDNF levels was detected with advancing age. Striatal levels of BDNF were stable from young adulthood through middle age but declined significantly in the oldest monkeys. These same monkey subjects have been assessed for

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biochemical and morphological markers of the nigrostriatal DA system, providing a context not available in the rat studies. Our studies show that striatal DA levels are significantly decreased by middle age in the intact striatum, and that THir neurons in substantia nigra exhibit no overt cell loss but do show decreasing soma size and intensity of TH immunoreactivity that is progressive from young adulthood through middle age into old age (Collier et al., 2003a,b). Thus, the increase in combined striatal trophic activity detected parallels the timing of degenerative changes in DA neurons and is likely to be a compensatory response triggered during the aging process in this system. Furthermore, our evidence suggests that chronic increases in striatal trophic activity generated during aging cannot be attributed to increases in GDNF or BDNF as these neurotrophic factors either do not change or decline. Finally, the trophic compensation generated at best appears to maintain a reduced level of striatal DA from middle age into old age and does not forestall progressive signs of morphological deterioration at the level of substantia nigra DA cell bodies.

The findings from the DA-depleted striatum of aging rats and monkeys must necessarily be interpreted in the context of the timing of sample collection following lesion. Both rat studies and our monkey study endeavored to examine trophic factor levels at a time believed to represent the characteristics of stable DA depletion and were meant to model the state of the striatum when therapeutic intervention might be instituted for a PD patient. Still, the scope of such analyses is limited by the required focus on selected time points. In rats, combined striatal trophic activity ipsilateral to DA depletion is significantly increased in younger animals (4 and 12 months old) and this response is entirely absent in older animals (18 and 23 months old) (Ling et al., 2000). This assay was performed at 8 weeks after unilateral 6-OHDA lesion. Striatal levels of BDNF and GDNF have been demonstrated to increase significantly at 2–4 weeks ipsilateral to unilateral DA depletion in young adult rats (4–5 months old), but no increase is detected in aged rats (31–34 months old) (Yurek and Fletcher-Turner, 2000, 2001). There is evidence that this 2- to 4-week timeframe may represent a period of maximal response for these factors, as BDNF levels while elevated in young adult rats at 2 weeks after DA depletion decline to baseline by 7 weeks after lesion (Zhao et al., 1996). Taken together, the evidence from aging rats supports the view that DA depletion triggers increased striatal trophic activity in young rats, but that this response is compromised in aged rats.

Consistent with findings in rats, young adult monkeys increased striatal trophic activity in response to severe DA depletion, while middle-aged and aged adults did not. This increase in young monkeys was sustained at 3 months after unilateral MPTP administration. The failure to detect an increase in trophic activity in older monkeys was potentially the result of the aged animals' inability to generate further increases beyond the already elevated baseline levels. Thus, the compensatory response in older monkeys may be saturated by the response to aging per se. Alternatively, we cannot rule out the possibility that older animals generate further increases in trophic activity over a transient time course. However, it is interesting that the combined striatal trophic response generated in young monkeys following sudden severe DA depletion is of the same magnitude as the response triggered by gradual aging-related deterioration of the DA system. This might favor the interpretation that the increase observed represents a biological maximum for this compensatory response.

Changes in BDNF and GDNF predicted by rat studies also did not hold true for monkeys. While young adult rats increased striatal BDNF levels at 2–4 weeks following DA depletion, young and middle-aged monkeys exhibited significant decreases in BDNF levels following DA depletion. Like aged rats, aged monkeys did not exhibit any change in BDNF following DA depletion. The equivalent levels of striatal BDNF displayed by DA-depleted young and middle-aged monkeys and the intact hemisphere of old monkeys is consistent with exhaustion of the pool of BDNF that resides within nigrostriatal DA neurons. The compensatory increase of BDNF seen in young rats following DA depletion is hypothesized to be a consequence of increased BDNF derived from non-DA neuron sources in the striatum (Yurek and Fletcher-Turner, 2000, 2001). The most likely source is provided by anterograde transport from cortex (Altar and DiStefano, 1998; Altar et al., 1997; Mufson et al., 1999). To the extent that this is accurate, our findings indicate that BDNF compensation from other sources either does not occur in nonhuman primates or occurs over a shorter time course and is not maintained. Our cases showed no significant change in striatal GDNF levels following DA depletion. While this indicates that any change in GDNF is not maintained over time, it does not rule out the possibility that a more transient response is generated as suggested by rat studies. Furthermore, these data indicate that any enhancement of endogenous GDNF that might be provoked by DA depletion is unlikely to be active at the time of therapeutic intervention in PD patients.

The disconnect between changes in striatal trophic activity during aging and in response to DA depletion in rats and monkeys was unanticipated but supports the importance of the nonhuman primate model for the final evaluation of experimental therapeutics for humans. The presence of elevated combined striatal trophic activity that is sustained in aging, and in response to MPTP-induced DA depletion, argues against the view that aging produces a generalized impoverished trophic environment that may adversely affect therapeutic strategies dependent upon this activity. However, we demonstrate that this increased trophic response is unable to forestall degenerative changes in the DA system, as these same monkeys display aging-related decreases in striatal DA and morphological signs of deterioration in nigral cell bodies. The combination of trophic molecules constituting this striatal response remains

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to be determined, but the failure of trophic signaling to completely preserve DA neuron integrity appears not to be a product of decreased levels of all molecules that can generate DA neurotrophic effects. Our findings suggest that chronic increases in striatal trophic activity expressed during aging and the compensatory response generated by DA depletion in young adult monkeys are not attributable to increased levels of BDNF or GDNF as these molecules either decreased or remained stable. This may suggest that declines in BDNF in conjunction with low adult levels of GDNF are specifically associated with aging-related deterioration of the DA system.

Acknowledgments

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